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(54) Title: PEGYLATION REAGENTS AND COMPOUNDS FORMED THEREWITH

(57) Abstract

Biologically active conjugates are disclosed which are formed by reaction of a thiol moiety of a biologically active molecule with a non-peptidic polymer having an active sulfone moiety. Also disclosed are compounds having the formula R_1-X-R_2 wherein at least one of R_1 and R_2 is a biologically active molecule having a reactive thiol moiety which forms a covalent bond with X, a Michael acceptor-activated non-peptidic polymer. Further disclosed are methods of making the conjugates and compounds of the present invention as well as pharmaceutical compositions containing them. In addition, activated polymers suitable for attachment to a variety of molecules and surfaces are disclosed.

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PEGYLATION REAGENTS AND COMPOUNDS FORMED THEREWITH

Field of the Invention

This invention relates to active derivatives of polyethylene glycol and related hydrophilic polymers and to methods for their synthesis for use in modifying the characteristics of surfaces and molecules. The invention also relates to polypeptides that have been covalently bonded to such active derivatives and methods for making the same.

Background of the Invention

Polyethylene glycol ("PEG") has been studied for use in pharmaceuticals, on artificial implants, and other applications where biocompatibility is of importance. Various derivatives of PEG have been proposed that have an active moiety for permitting PEG to be attached to pharmaceuticals and implants and to molecules and surfaces generally. For example, PEG derivatives have been proposed for coupling PEG to surfaces to control wetting, static buildup, and attachment of other types of molecules to the surface, including proteins or protein residues.

PEG derivatives have also been proposed for affinity partitioning, for example, of enzymes from a cellular mass. In affinity partitioning, the PEG derivative includes a functional group for reversible coupling to an enzyme that is contained within a cellular mass. The PEG and enzyme conjugate is separated from the cellular mass and then the enzyme is separated from the PEG derivative, if desired.

In still further examples, coupling of PEG derivatives ("PEGylation") is desirable to overcome obstacles encountered in the clinical use of biologically active molecules. Published PCT Publication No. WO 92/16221 states, for example, that many potentially therapeutic proteins have been found to have a short half life in the blood serum. For the most part, proteins are cleared from the serum through the kidneys. The systematic introduction of relatively large quantities of proteins, particularly those foreign to the human system, can give rise to immunogenic reactions that, among other problems, may lead to rapid removal of the protein from

the body through formation of immune complexes. For other proteins, solubility and aggregation problems have also hindered the optimal formulation of the protein.

PEGylation decreases the rate of clearance from the bloodstream by increasing the apparent molecular weight of the molecule. Up to a certain size, the rate of glomerular filtration of proteins is inversely proportional to the size of the protein. The ability of PEGylation to decrease clearance, therefore, is generally not a function of how many PEG groups are attached to the protein, but the overall molecular weight of the altered protein. Decreased clearance can lead to increased efficiency over the non-PEGylated material. See, for example, Conforti et al., Pharm. Research Commun. vol. 19, pg. 287 (1987) and Katre et al., Proc. Natl. Acad. Sci. U.S.A. vol. 84, pg. 1487 (1987).

In addition, PEGylation can decrease protein aggregation (Suzuki et al., Biochem. Biophys. Acta vol. 788, pg. 248 (1984)), alter protein immunogenicity (Abuchowski et al., J. Biol. Chem. vol. 252 pg. 3582 (1977)), and increase protein solubility as described, for example, in PCT Publication No. WO 92/16221.

PEGylation of proteins illustrates some of the problems that have been encountered in attaching PEG to surfaces and molecules. The vast majority of PEGylating reagents react with free primary amino groups of the polypeptide. Most of these free amines are the epsilon amino group of lysine amino acid residues. Typical proteins possess a large number of lysines. Consequently, random attachment of multiple PEG molecules often occurs leading to loss of protein activity.

In addition, if the PEGylated protein is intended for therapeutic use, the multiple species mixture that results from the use of non-specific PEGylation leads to difficulties in the preparation of a product with reproducible and characterizable properties. This non-specific PEGylation makes it difficult to evaluate therapeutics and to establish efficacy and dosing information. The site selective PEGylation of such proteins could lead to reproducibly-modified materials that gain the desirable attributes of PEGylation without the loss of activity.

The need to reproducibly create complexes of two or more linked bioactive molecules or compounds also exists. In certain cases, the administration of

multimeric complexes that contain more than one biologically active polypeptide or drug leads to synergistic benefits. For example, a complex containing two or more identical binding polypeptides may have substantially increased affinity for the ligand or active site to which it binds relative to the monomeric polypeptide. Alternatively, a complex comprised of (1) a bioactive protein that exerts its effect at a particular site in the body and (2) a molecule that can direct the complex to that specific site may be particularly beneficial.

A need also exists for hydrolytically-stable activated polymers which form linkages which are also hydrolytically stable. Otherwise, in certain cases, the reactive group can be rendered inactive before the desired reaction takes place or the conjugate formed after reaction has a short half life in aqueous media, such as blood or plasma.

For example, Zalipsky U.S. Patent No. 5,122,614 describes that PEG molecules activated with an oxycarbonyl-N-dicarboximide functional group that can be attached under aqueous, basic conditions by a urethane linkage to the amine group of a polypeptide. Activated PEG-N-succinimide carbonate is said to form stable, hydrolysis-resistant urethane linkages with amine groups. The amine group is shown to more reactive at basic pHs of about 8.0 to 9.5, and reactivity falls off sharply at lower pHs. Hydrolysis of the uncoupled PEG derivative, however, also increases sharply at pHs of 8.0 to 9.5. Zalipsky avoids the problem of an increase in the rate of reaction of the uncoupled PEG derivative with water by using an excess of PEG derivative to bind to the protein. By using an excess of PEG derivative, sufficient reactive amino sites are bound to PEG to modify the protein before the PEG derivative becomes hydrolyzed and unreactive.

Zalipsky's method is adequate for nonspecific attachment of the lysine fraction of a protein to a PEG derivative at one active site on the PEG. If the rate of hydrolysis of the PEG derivative is substantial, however, then it can be problematic to provide attachment at more than one active site on the PEG molecule, since a simple excess does not slow the rate of hydrolysis.

For example, a linear PEG with active sites at each end will attach to protein at one end but the reactive site at the other end can react with water to form a relatively nonreactive hydroxyl moiety instead of a PEG linking two protein groups. A similar problem arises if it is desired to couple a molecule to a surface by a PEG linking agent because the PEG is first attached to the surface or couples to the molecule, and the opposite end of the PEG derivative must remain active for a subsequent reaction. If hydrolysis is a problem, then the opposite end typically becomes inactivated.

Zalipsky U.S. Patent No. 5,122,614 also describes several other PEG derivatives from prior patents. PEG-succinoyl-N-hydroxysuccinimide ester is said to form ester linkages that have limited stability in aqueous media. PEG-cyanuric chloride is said to be toxic and is non-specific for reaction with particular functional groups on a protein which can lead to protein inactivation. PEG-phenylcarbonate is said to produce toxic hydrophobic phenol residues that have an affinity for proteins. PEG activated with carbonyldiimidizole is said to be too slow in reacting with protein functional groups, requiring long reaction times to obtain sufficient modification of the protein.

Still other PEG derivatives have been proposed for attachment to functional groups other than the epsilon amino group of lysine. Maleimide, for example, is specific for cysteine sulfhydryl but the maleimide functionality is subject to hydrolysis.

Accordingly, a need exists for reagents and methods for reproducibly creating complexes whose parts are linked by nonantigenic, highly soluble, biologically inert molecules. The present invention satisfies the need for such complexes and provides related advantages. The present invention also satisfies the need for hydrolytically stable reagents that form hydrolytically stable conjugates.

Summary of the Invention

The present invention relates to biologically-active conjugates containing a biologically-active molecule having a reactive thiol moiety and a non-peptidic polymer

having an active sulfone moiety which forms a link with the reactive thiol moiety. The biologically-active molecule can be a synthetic, a naturally occurring, or a modified naturally occurring molecule. A molecule possessing the desired biological activity can be modified to contain a reactive thiol moiety.

Particularly useful biologically active molecules include the tumor necrosis factor ("TNF') inhibitors, Interleukin-1 receptor antagonists ("IL-1ra's"), CR1, exon six peptide of PDGF, and the Interleukin-2 ("IL-2") inhibitors and receptors ("IL-2r").

The polymer of the present invention contains at least one active sulfone moiety and has the formula P-SO_2-C-C*- , where P is polymer and C* is a reactive site for linkage with thiol moieties. The link between the thiol and activated sulfone is at C* and can be represented by the formula P-SO_2-C-C*S-R, where R is the biologically-active molecule. Useful activated sulfone moieties include, for example, vinyl sulfone and chloroethyl sulfone. Various polymers can be activated for use in all embodiments of the present invention including water soluble polymers such as polyethylene glycol ("PEG") and related hydrophilic polymers.

The present invention also provides methods of using sulfone-activated polymers to make the biologically-active conjugates discussed above. The method includes the steps of:

(a) reacting the biologically-active molecule having a reactive thiol moiety with a non-peptidic polymer having an active sulfone moiety to form a conjugate; and

(b) isolating the conjugate.

Pharmaceutical compositions containing the conjugates are also within the scope of the invention.

The present invention further relates to sulfone-activated polymers useful for coupling to a variety of molecules, compounds, and surfaces. The activated sulfone moiety is the same as discussed above. Particularly useful activated polymers include bifunctional PEG derivatives activated with a sulfone moiety at one site on the PEG molecule and an NHS-ester or a maleimide functionality at another site.

Further included in the present invention are substantially purified biologically-active compounds having the formula R_1-X-R_2 , called a "dumbbell" where at least one of R_1 or R_2 is a biologically-active molecule which retains its biological activity when part of the compound. The biologically-active molecule has a reactive thiol moiety which forms a link with a Michael acceptor group on a non-peptidic polymer. Biologically-active molecules suitable for use in the present invention include those mentioned above. Useful Michael acceptor groups include, for example, vinyl sulfone and maleimide. Polymers which can be activated with Michael acceptor functional groups include the water soluble polymers mentioned above.

R_1 and R_2 can be the same or different moieties. When the R groups are the same, the compound is a homodumbbell; when the R groups are different, the compound is a heterodumbbell. Particularly useful homodumbbells include, for example, PEG-linked TNF inhibitors and PEG-linked IL-1ra's. Useful heterodumbbells include, for example, those formed from IL-2r-α and IL-2r-β, heterodumbbells which inhibit the classical pathway of the complement system, and heterodumbbells formed from IL-1ra and exon 6 of PDGF.

Methods of making the dumbbell compounds are within the scope of the invention. The methods of making a dumbbell, R_1-X-R_2, include the steps of:

(a) reacting X with R_1 and R_2 to form R_1-X-R_2; and

(b) purifying R_1-X-R_2.

Step (a) in the above methods of making dumbbells can further include the following steps:

protecting one reactive group of X to form a protected group on X;

reacting X having a protected group with R_1 to form R_1-X;

deprotecting the protected group on X; and

reacting R_1-X with R_2 to form R_1-X-R_2.

Alternatively or in addition, step (a) can further include the following steps:

reacting an excess of X with R_1 to form R_1-X; and

reacting R_1-X with R_2 to form R_1-X-R_2.

Pharmaceutical compositions containing the substantially purified compounds R_1-X-R_2 are also within the scope of the invention.

Detailed Description

The present invention provides biologically-active conjugates containing (1) a biologically-active molecule having a reactive thiol moiety, and (2) a non-peptidic polymer having an active sulfone moiety which forms a linkage with the thiol moiety of the biologically-active molecule.

A "conjugate" means a complex that is formed by joining a biologically-active molecule, having an active thiol moiety, to a non-peptidic polymer, having an active sulfone moiety, via a linkage between the thiol and sulfone. As stated above, the conjugates of the present invention are biologically active.

"Biologically active" means capable of exerting a biological effect, in vitro or in vivo. A biologically active molecule includes, but is not limited to, any compound that can induce a biological effect on interaction with a natural biological molecule or on a biological system such as a cell or organism. Ways of demonstrating biological activity include in-vitro bioassays, many of which are well known in the art. For example, one can measure the biological activity of tumor necrosis factor ("TNF") inhibitors by determining if the inhibitors bind to TNF or if the inhibitors block TNF-mediated lysis of certain cells. The latter bioassay is set forth in published European Patent Application No. 90113673.9, which is specifically incorporated herein by reference.

Biologically-active molecules include, but are not limited to, pharmaceuticals, vitamins, nutrients, nucleic acids, amino acids, polypeptides, enzyme co-factors, steroids, carbohydrates, organic species such as heparin, metal containing agents, receptor agonists, receptor antagonists, binding proteins, receptors or portions of receptors, extracellular matrix proteins, cell surface molecules, antigens, haptens, targeting groups, and chelating agents. All references to receptors include all forms of the receptor whenever more than a single form exists.

"Polypeptides" and "proteins" are used herein synonymously and mean any compound that is substantially proteinaceous in nature. However, a polypeptidic group may contain some non-peptidic elements. For example, glycosylated polypeptides or synthetically modified proteins are included within the definition. "Targeting groups" can direct a compound to a location in a biological system. Binding proteins and receptors can be described by their affinity for a certain ligand.

Many polypeptides useful in the present invention are set forth in published PCT Publication No. WO 92/16221, specifically incorporated herein by reference. These proteins are well known in the art. Particularly useful polypeptides are the TNF binding proteins, also called TNF inhibitors. A "TNF binding protein" is defined herein to mean a protein that binds TNF.

One TNF binding protein ("TNFbp") is the extracellular portion of the p55 TNF receptor or the TNF receptor I. In vivo, the extracellular portion of the receptor is shed and circulates in the bloodstream as a 30kDa glycosylated protein which binds to TNF. This binding protein is also referred to TNFbp-I or the 30kDa TNFbp. The purification and amino acid and nucleic acid sequences of this TNF binding protein are set forth in published European Patent Application No. 90 113 673.9, which is incorporated herein by reference.

This published reference also teaches the recombinant production of glycosylated and deglycosylated forms of this TNF inhibitor. Although the actual molecular weight of the deglycosylated form of this inhibitor is approximately 18kDa, the term "30kDa TNF inhibitor" includes the glycosylated and deglycosylated forms.

As used herein, the terms "naturally-occurring," "native," and "wild-type" are synonymous.

European Patent Application No. 90 113 673.9, incorporated herein by reference, also sets forth the purification and amino acid and nucleic acid sequences of another TNF inhibitor, called the 40kDa TNF inhibitor. Also called TNFbp-II, this inhibitor, in its naturally-occurring form, is the glycosylated extracellular portion of the p75 or p85 TNF receptor. European Patent Application No. 90 112 673.9 also teaches the recombinant production of the glycosylated and deglycosylated forms of

this "40kDa" inhibitor. The nucleic and amino acid sequences of the native 40kDa TNF inhibitor are set forth in this published reference. Although the molecular weight of the deglycosylated form is not 40kDa, both the glycosylated and deglycosylated forms of this TNFbp are referred to as "40kDa TNF inhibitor."

European Patent Application No. 90 112 673.9, incorporated herein by reference, further teaches the recombinant production of two TNF inhibitors which are portions of the full length "40kDa" binding protein. These two truncates are called the "Δ51" and "Δ53" TNF inhibitors. The amino acid and nucleic acid sequences of the Δ51 and Δ53 inhibitors are set forth in this published reference.

Other particularly useful polypeptides include the interleukin-1 receptor antagonists ("IL-1ra's"), as described in U.S. Patent No. 5,075,222, incorporated herein by reference, insulin-like growth factor binding proteins ("IGFbps"), CTLA4, and exon six of platelet derived growth factor ("PDGF"), glial derived neurotrophic factor ("GDNF"), ciliary neurotrophic factor ("CNTF"), interleukin-4 receptor ("IL-4r), and inhibitors, and interleukin-1 receptor ("IL-2r"). The nucleic acid encoding the naturally occurring IL-1ra and a method for expressing the protein in E. Coli. are set forth in United States Patent No. 5, 075, 222 of Hannum et al.

Characteristics of the IL-2 receptors and CR1, the nucleic acids encoding them, and methods for their production are discussed in published PCT Publication No. WO 92/16221, specifically incorporated herein by reference.

The biologically-active molecules linked to polymers in the conjugates of the present invention have a reactive thiol moiety prior to forming the linkage. A "reactive thiol moiety" means a -SH group capable of reacting with the activated polymers as described herein.

An example of a reactive thiol is the -SH of the amino acid cysteine. Many proteins do not have free cysteines (cysteines not involved in disulfide bonding) or any other reactive thiol group. In addition, the cysteine thiol may not be appropriate for linkage to the polymer because the thiol is necessary for biological activity. In addition, proteins must be folded into a certain conformation for activity. In the active conformation, a cysteine can be inaccessible for reaction with sulfone because

it is buried in the interior of the protein. Moreover, even an accessible cysteine thiol which is not necessary for activity can be an inappropriate site to form a linkage to the polymer. Amino acids not essential for activity are termed "nonessential." Nonessential cysteines can be inappropriate conjugation sites because the cysteine's position relative to the active site results in the polypeptide becoming inactive after conjugation to polymer. Like proteins, many other biologically-active molecules have reactive thiols which, for reasons similar to those recited above, are not suitable for conjugation to the polymer or contain no reactive thiol groups.

Accordingly, the present invention contemplates the introduction of reactive thiol groups into a biologically-active molecule when necessary or desirable. Thiol groups can also be introduced into an inactive molecule to form a biologically-active molecule as long as the thiol-sulfone link does not destroy the desired activity.

Reactive thiol groups can be introduced by chemical means well known in the art. Chemical modification can be used with polypeptides or non-peptidic molecules and includes the introduction of thiol alone or as part of a larger group, for example a cysteine residue, into the molecule. An example of chemically introducing thiol is set forth in Jue, R. et al., Biochemistry, 17, pp. 5399-5406 (1978). One can also generate a free cysteine in a polypeptide by chemically reducing cystine with, for example, DTT.

Polypeptides which are modified to contain an amino acid residue in a position where one was not present in the native protein before modification is called a "mutein." To create cysteine muteins, a nonessential amino acid can be substituted with a cysteine or a cysteine residue can be added to the polypeptide. Potential sites for introduction of a non-native cysteine include glycosylation sites and the N or C terminus of the polypeptide. The mutation of lysine to cysteine is also appropriate because lysine residues are often found on the surface of a protein in its active conformation. In addition, one skilled in the art can use any information known about the binding or active site of the polypeptide in the selection of possible mutation sites.

One skilled in the art can also use well known recombinant DNA techniques to create cysteine muteins. One can alter the nucleic acid encoding the native polypeptide to encode the mutein by standard site directed mutagenesis. Examples of standard mutagenesis techniques are set forth in Kunkel, T.A., Proc. Nat. Acad. Sci., Vol. 82, pp. 488-492 (1985) and Kunkel, T.A. et al., Methods Enzymol., Vol. 154, pp. 367-382 (1987), both of which are incorporated herein by reference. Alternatively, one can chemically synthesize the nucleic acid encoding the mutein by techniques well known in the art. DNA synthesizing machines can be used and are available, for example, from Applied Biosystems (Foster City, CA). The nucleic acid encoding the desired mutein can be expressed in a variety of expression systems, including animal, insect, and bacterial systems.

When the mutein is recombinantly produced in a bacterial expression system, the following steps are performed:

1) The nucleic acid encoding the desired mutein is created by site directed mutagenesis of the nucleic acid encoding the native polypeptide;

2) The nucleic acid encoding the desired mutein is expressed in a bacterial expression system;

3) The mutein is isolated from the bacteria and purified;

4) If not folded properly, the mutein is refolded in the presence of cysteine or another sulphydryl containing compound;

5) The refolded mutein is isolated and purified;

6) The purified and refolded target mutein is treated with a mild reducing agent;

7) The reaction mixture is dialyzed in the absence of oxygen.

As discussed below, the mutein can be isolated from the reaction mixture prior to conjugation with polymer but need not be. A reducing agent particularly useful in step 6 is dithiothreitol ("DTT") or Tris-(carboxyethylphosphine) ("TCEP"). TCEP is useful because it does not have to be removed before conjugation with a thiol-specific PEG reagent. See Burns, J.A. et al., J. Org. Chem., Vol.56, No. 8, pp. 2648-2650 (1991).

After creation of the desired mutein, one skilled in the art can bioassay the mutein and compare activity of the mutein relative to the native polypeptide. As more fully discussed below, even if the relative activity of the mutein is diminished, the conjugate formed from the mutein can be particularly useful. For example, the conjugate can have increased solubility, reduced antigenicity or immunogenicity, or reduced clearance time in a biological system relative to the unconjugated molecule. Such improvements in the pharmacokinetic performance of the biologically-active molecule can increase the molecule's value in various therapeutic applications. Increased solubility can also improve the value of the molecule for in-vitro diagnostic applications.

Table 1 lists muteins of IL-1ra that have been produced. The preparation and purification of IL-1ra muteins are set forth in published PCT Patent Publication No. WO 92/16221, specifically incorporated herein by reference. The residue numbering is based upon the sequence set forth in that published application with "0" denoting addition of an amino acid at the N-terminus; "c" referring to cysteine and "s" referring to serine. For example, "c0s116" means a cysteine was inserted at the N terminus and a serine was inserted at position 116. Native IL-1ra has free cysteine residues at positions 66, 69, 116 and 122.

TABLE 1. MUTEINS OF IL-1ra

c0s116	c0
c84s116	c6
c8s116	c8
c9s116	c9
c141s116	c141

Table 2 shows muteins of the 30kDa TNF inhibitor which have also been prepared. The native 30kDa TNF inhibitor, unlike IL-1ra, does not have any free

cysteine residues. These muteins have been prepared as set forth in published PCT Publication No.
WO 92/16221, specifically incorporated herein by reference, and the numbering is based upon the amino acid sequence set forth therein.

TABLE 2. MUTEINS OF 30kDa TNF INHIBITOR

c105 30kDa TNF inhibitor
c1 30kDa TNF inhibitor
c14 30kDa TNF inhibitor
c111 30kDa TNF inhibitor
c161 30kDa TNF inhibitor

The muteins and other polypeptides of the present invention include allelic variations in the protein sequence and substantially equivalent proteins. "Substantially equivalent," means possessing a very high degree of amino acid residue homology (See generally, M. Dayhoff, Atlas of Protein Sequence and Structure, vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by references) as well as possessing comparable biological activity. Also included within the scope of this invention are truncated forms of the native polypeptide or mutein that substantially retain the biological activity of the native polypeptide or mutein.

The conjugates of the present invention contain, in addition to biologically-active molecules having reactive thiol moieties, non-peptidic polymeric derivatives having active sulfone moieties. "Non-peptidic" means having less than 50% by weight of α amino acid residues.

The polymer portion of the polymeric derivative can be, for example, polyethylene glycol ("PEG"), polypropylene glycol ("PPG"), polyoxyethylated glycerol ("POG") and other polyoxyethylated polyols, polyvinyl alcohol ("PVA") and

other polyalkylene oxides, polyoxyethylated sorbitol, or polyoxyethylated glucose. The polymer can be a homopolymer, a random or block copolymer, a terpolymer based on the monomers listed above, straight chain or branched, substituted or unsubstituted as long as it has at least one active sulfone moiety. The polymeric portion can be of any length or molecular weight but these characteristics can affect the biological properties. Polymer average molecular weights particularly useful for decreasing clearance rates in pharmaceutical applications are in the range of 2,000 to 35,000 daltons. In addition, if two groups are linked to the polymer, one at each end, the length of the polymer can impact upon the effective distance, and other spatial relationships, between the two groups. Thus, one skilled in the art can vary the length of the polymer to optimize or confer the desired biological activity. If the polymer is a straight chain PEG, particularly useful lengths of polymers, represented by $(Z)_n$, where Z is the monomeric unit of the polymer, include n having a range of 50-500. In certain embodiments of the present invention, n is greater than 6 and preferably greater than 10.

Monomethoxy polyethylene glycol is designated here as mPEG. The term "PEG" means any of several condensation polymers of ethylene glycol. PEG is also known as polyoxyethylene, polyethylene oxide, polyglycol, and polyether glycol. PEG can also be prepared as copolymers of ethylene oxide and many other monomers. For many biological or biotechnical applications, substantially linear, straight-chain vinyl sulfone activated PEG will be used which is substantially unsubstituted except for the vinyl sulfone.

PEG is useful in biological applications for several reasons. PEG typically is clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze, and is nontoxic. PEGylation can improve pharmacokinetic performance of a molecule by increasing the molecule's apparent molecular weight. The increased apparent molecular weight reduces the rate of clearance from the body following subcutaneous or systemic administration. In many cases, PEGylation can decrease antigenicity and immunogenicity. In addition, PEGylation can increase the solubility of a biologically-active molecule.

The polymeric derivatives of the present invention have active sulfone moieties. "Active sulfone" means a sulfone group to which a two carbon group is bonded having a reactive site for thiol-specific coupling on the second carbon from the sulfone group at about pH 9 or less. Examples of active sulfones include, but are not limited to, vinyl sulfone and activated ethyl sulfone. An example of an active ethyl sulfone is $-SO_2-CH_2-CH_2-Z$ where Z is halogen or another leaving group capable of substitution by thiol to form the sulfone-thiol linkage $-SO_2-CH_2-CH_2-R$, where R represents a biologically active molecule. The sulfone-activated polymer can be further substituted as long as the thiol-specific reactivity at the second carbon is maintained at about pH 9 or less.

The sulfone-activated polymers of the present invention can be synthesized in at least four steps. Briefly, the first step is to increase the reactivity of a site on the polymer, typically an end group, by, for example, activation or substitution. The second step is to link sulfur directly to a carbon atom in the polymer in a form that can be converted to an ethyl sulfone or ethyl sulfone derivative having similar reactive properties. In the third step, the sulfur is oxidized to sulfone. In the fourth step, the second carbon from the sulfone group is activated.

The synthesis of a sulfone-activated polymer is described in more detail below using the synthesis of a sulfone-activated PEG as an example. The first step is the hydroxyl activation of an hydroxyl moiety in the PEG. The term "hydroxyl activation" should be interpreted herein to mean substitution as well as esterification and other methods of hydroxyl activation. Typically, in hydroxyl activation, an acid or an acid derivative such as an acid halide is reacted with the PEG to form a reactive ester in which the PEG and the acid moiety are linked through the ester linkage. The acid moiety generally is more reactive than the hydroxyl moiety. Typical esters are the sulfonate, carboxylate, and phosphate esters.

Sulfonyl acid halides that are suitable for use in the invention include, for example, methanesulfonyl chloride (also known as mesyl chloride) and *p*-toluene-sulfonyl chloride (also known as tosyl chloride). Methanesulfonate esters are

sometimes referred to as mesylates. Toluenesulfonate esters are sometimes referred to as tosylates.

In a substitution type of hydroxyl activation, the entire hydroxyl group on the PEG is substituted by a more reactive moiety, typically a halide. For example, thionyl chloride, can be reacted with PEG to form a more reactive chlorine substituted PEG.

Thus, when PEG is the starting material, the typical reaction product of the first step is an ester or halide-substituted PEG.

In the second step, the ester or halide is substituted by an alcohol which contains a reactive thiol attached to an ethyl group, a thioethanol moiety. Thioethanol is an example of a suitable alcohol. In this step, the sulfur in the thiol is bonded directly to a carbon on the polymer.

Next, in the third step, the sulfur is oxidized to sulfone. Useful oxidizing agents include, for example, hydrogen peroxide, sodium perborate, or peroxy acids.

In the fourth step, the hydroxyl moiety of the alcohol used in step two is activated. This step is similar to the first step in the reaction sequence. Substitution typically is with halide to form a haloethyl sulfone or a derivative thereof having a reactive site on the second carbon removed from the sulfone moiety. Typically, the second carbon on the ethyl group will be activated by a chloride or bromide halogen. Hydroxyl activation should provide a site of similar reactivity, such as the sulfonate ester. Suitable reactants are, for example, the acids, acid halides, and others previously mentioned in discussing the first step in the reaction. Thionyl chloride is particularly useful for substitution of the hydroxyl group with the chlorine atom.

The resulting polymeric activated ethyl sulfone is stable, isolatable, and suitable for thiol-selective coupling reactions. PEG chloroethyl sulfone is stable in water at a pH of about 7 or less, but nevertheless can be used to advantage for thiol-selective coupling reactions at conditions of basic pH up to at least about pH 9. At a pH of above about 9, the thiol selectivity is diminished and the sulfone moiety becomes somewhat more reactive with amino groups. The linkage formed upon reaction with thiol is also hydrolytically stable.

In a fifth step that can be added to the synthesis, the activated ethyl sulfone is reacted with a base to from PEG vinyl sulfone or one of its active derivatives for thiol-selective coupling. Suitable bases include, for example, sodium hydroxide or triethylamine. Like activated ethyl sulfones, vinyl sulfone is hydrolytically stable, isolatable, thiol-selective, and forms hydrolytically-stable linkages upon reaction with thiol.

As used herein, "hydrolytically stable" means that the linkage between the polymer and the sulfone moiety and between the sulfone-thiol after conjugation does not react with water at a pH of less than about 11 for at least three days. Hydrolytic stability is desirable because, if the rate of hydrolysis is significant, the polymer can be deactivated before the reaction between polymer and the thiol of the biologically-active molecule takes place.

As mentioned above, for example, a linear PEG with active sites at each end will attach to a protein at one end, but, if the rate of hydrolysis is significant, will react with water at the other end to become capped with a relatively nonreactive hydroxyl moiety, rather than forming a "dumbbell" molecular structure with attached proteins or other desirable groups on each end. A similar problem arises when coupling a molecule to a surface by a PEG linking agent because the PEG is first attached to the surface or couples to the molecule, and the opposite end of the PEG derivative must remain active for a subsequent reaction. If hydrolysis is a problem, then the opposite end typically becomes inactivated.

Alternatively, the sulfone-activated derivatives can be prepared by attaching a linking agent having a sulfone moiety to a PEG (or other polymer) activated with a different functional group. For example, an amino activated PEG can be reacted under favorable conditions of pH of about 9 or less with a small molecule that has a succinimidyl active ester moiety at one terminus and vinyl sulfone at the other terminus. The amino-activated PEG forms a stable linkage with the succinimidyl ester. The resulting PEG is activated with the vinyl sulfone at the terminus and is hydrolytically stable: PEG-NH-OC-CH_2-CH_2-$SO_2CH=CH_2$.

A similar activated PEG can be achieved by reacting an amine-reactive PEG such as succinimidyl active ester PEG, PEG-CO_2-NHS, with a small molecule that has an amine moiety at one terminus and a vinyl sulfone moiety at the other terminus.

PEG chloroethyl sulfone and PEG vinyl sulfone were prepared as set forth in Example 1. Thiol-selective reactivity of PEG vinyl sulfone and chloroethyl sulfone is shown in Example 2. Hydrolytic stability of the polymer-sulfone linkage of two compounds is shown in Example 3. Hydrolytic stability of the linkage between thiol and sulfone is shown in Example 16.

When the polymer does not have an hydoxyl moiety, one can first be added by chemical methods well known in the art before carrying out the steps described above. The activated polymeric derivatives of the present invention can have more than one reactive group. The derivatives can be monofunctional, bifunctional, or multifunctional. The reactive groups may be the same (homofunctional) or different (heterofunctional) as long as there is at least one active sulfone moiety.

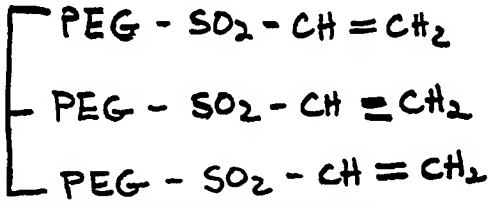
Two particularly useful homobifunctional derivatives are PEG-*bis*-chlorosulfone and PEG-*bis*-vinyl sulfone. One skilled in the art can synthesize those molecules using PEG having hydroxyl moieties at each end as a starting material and following the general method set forth above.

Heterobifunctional derivatives can also be synthesized. Two particularly useful heterobifunctional derivatives include, for example, a linear PEG with either a vinyl sulfone or a maleimide at one end and an N-hydroxysuccinimide ester ("NHS-ester") at the other end. The NHS-ester is amine-specific. PEG having an NHS-ester at one end and an activated sulfone moiety at the other can be attached to both lysine and cysteine residues. A stable amine linkage can be achieved, leaving the hydrolytically-stable unreacted sulfone available for subsequent reaction with thiol. Those two heterobifunctional PEG derivatives have been synthesized as described in Examples 5 and 6. If the maleimide NHS-ester heterobifunctional reagent is made using straight-chain PEG, represented by $(Z)_n$, where Z is the monomeric unit, n is greater than 6 and preferably greater than 10.

Other active groups for heterofunctional sulfone-activated PEGs can be selected from among a wide variety of compounds. For biological and biotechnical applications, the substituents would typically be selected from reactive moieties typically used in PEG chemistry to activate PEG such as the aldehydes, trifluoroethylsulfonate (sometimes called tresylate), n-hydroxylsuccinimide ester, cyanuric chloride, cyanuric fluoride, acyl azide, succinate, the *p*-diazo benzyl group, the 3-(*p*-diazophenyloxy)-2-hydroxy propyloxy group, and others.

Examples of active moieties other than sulfone are shown in Davis et al. U.S. Patent No. 4,179,337; Lee et al. U.S. Patent Nos. 4,296,097 and 4,430,260; Iwasaki et al. 4,670,417; Katre et al. U.S. Patent Nos. 4,766,106; 4,917,888; and 4,931,544; Nadagawa et al. U.S. Patent No. 4,791,192; Nitecki et al. U.S. Patent No. 4,902,502 and 5,089,261; Saifer U.S. Patent No. 5,080,891; Zalipsky U.S. Patent No. 5,122,614; Shadle et al. U.S. Patent No. 5,153, 265; Rhee et al. U.S. Patent No. 5,162,430; European Patent Application Publication No. 0 247 860; and PCT International Application Nos. US86/01252; GB89/01261; GB89/01262; GB89/01263; US90/03252; US90/06843; US91/06103; US92/00432; and US92/02047, the contents of which are incorporated herein by reference.

An example of a trifunctional derivative is a glycerol backbone to which three vinyl sulfone PEG moieties are attached. This molecule can be represented by the formula:



This derivative was prepared as described in Example 12.

Another example of a mutifunctional derivative is the "star" molecule. Star molecules are generally described in Merrill U.S. Patent No. 5,171,264, incorporated herein by reference. Star molecules have a core structure to which multiple PEG chains or "arms" are attached. The sulfone moieties can be used to provide an active, functional group on the end of the PEG chain extending from the core and as a linker for joining a functional group or other moiety to the star molecule arms.

It should be apparent to the skilled artisan that the activated polymers discussed above could be used to carry a wide variety of substituents and combinations of substituents.

As stated above, the conjugates of the present invention are formed by reacting thiol-containing biologically-active molecules with sulfone-activated polymers. The linkage between the thiol reactive group and the sulfone-activated polymer is a covalent bond.

A general method for preparing the conjugates of the present invention includes the following steps:

(1) Choose the desired biologically-active molecule and determine if the molecule possesses a free thiol group by means well known in the art. See, for example, Allen, G., "Sequencing of Proteins and Peptides," pp. 153-54, in Laboratory Techniques in Biochemistry and Molecular Biology, Work, T.S., and Burdon, R.H., eds. (1972), incorporated herein by reference. If the molecule has a free thiol, proceed to step 3. If the molecule has no free thiol, proceed to step 2.

(2) If no free thiol exists in the molecule, add thiol as discussed above. After adding thiol, perform a bioassay to determine if the desired biological activity or a portion of the biological activity is retained.

(3) Synthesize the desired sulfone-activated polymer as discussed above.

(4) React the activated polymer with the molecule having a free thiol.

(5) Isolate the reaction product using chromatographic techniques well known in the art. For protein conjugates, see, for example, Scopes, R., Protein Purification, Cantor, C.R. ed., Springer-Verlag, New York (1982). For nonprotein molecules, see, for example, Still, W.C. et al., J. Org. Chem., 43, pp.2923-2925 (1978). If no conjugate forms, add thiol to another location on the biologically-active molecule and repeat steps (4) and (5).

(6) Determine biological activity of the conjugate formed using the relevant bioassay.

One skilled in the art can add or delete certain steps. For example, one skilled in the art might not assay bioactivity in step 2 or might presume biological activity

after PEGylation based upon previous experiments. The skilled artisan can also add the step of varying the size, length, or molecular weight of the linker to optimize or confer biological activity.

Several conjugates have been prepared. The 30kDa TNFbp c105 mutein described above was conjugated with PEG vinyl sulfone as described in Example 10. Example 8 shows that native IL-1ra, which contains four free cysteines, reacted under similar conditions. The c84 IL-1ra mutein also reacted well. Example 13 shows the conjugation of three 30kDa TNF inhibitor muteins to three PEG chains bonded to a glycerol backbone.

The conjugates of the present invention can be used for a variety of purposes including, but not limited to, in-vitro diagnostic assays and the preparation of pharmaceutical compositions. Many of the conjugates of the present invention have at least one of the following characteristics relative to the unconjugated molecule:

(1) increased solubility in aqueous solution;

(2) reduced antigenicity or immunogenicity;

(3) reduced rate of clearance following subcutaneous or systemic administration due to increased apparent molecular weight.

Pharmaceutical preparations of conjugates containing IL-1ra are particularly useful. IL-1ra, alone or in combination with the 30kDa TNF binding protein, can be used to treat arthritis, inflammatory bowel disease, septic shock, ischemia injury, reperfusion injury, osteoporosis, asthma, insulin diabetes, myelogenous and other leukemias, psoriasis, adult respiratory distress syndrome, cachexia/anorexia, and pulmonary fibrosis.

Conjugates containing TNF binding proteins ("TNFbps") are also particularly useful. Such conjugates can be used to treat TNF-mediated diseases such as adult respiratory distress syndrome, pulmonary fibrosis, arthritis, septic shock, inflammatory bowel disease, multiple sclerosis, graft rejection and hemorrhagic trauma.

The biologically active conjugates of the present invention can further include non-biologically active moieties.

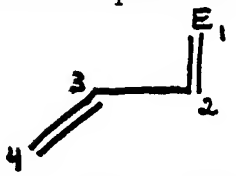
The present invention also includes substantially purified compounds having the formula R_1-X-R_2, where at least one of R_1 and R_2 is a biologically-active molecule having a reactive thiol moiety which forms a covalent bond with X, a Michael acceptor-activated polymer. In the present invention, the biological activity of R_1-X-R_2 retains the biological activity of R_1 or R_2. Molecules having the formula R_1-X-R_2 are referred to herein as "dumbbell" molecules.

As stated above, the compounds of the present invention are substantially purified. "Substantially purified" as used herein means a "homogenous composition." A homogenous composition contains molecules of R_1-X-R_2 and is substantially free from compounds that (1) deviate in the composition of R_1 or R_2, or (2) are linked together by more than one activated polymer. The homogeneous composition can contain molecules of R_1-X-R_2 which differ in the length of X. For straight-chain polymers, represented by $(Z)_n$, where Z is the monomeric unit, n is greater than 6 and preferably greater than 10. To have a homogeneous composition, R_1 and R_2 need not be attached to X at the same location on X or on the same location on either R group.

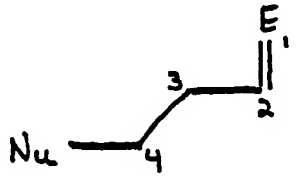
X is a non-peptidic polymer having a first reactive group and a second reactive group. A "reactive group" is a group capable of reacting with R. At least one reactive group on X is a Michael-type acceptor. The terms "reactive group" and "functional group" are used herein synonymously. The terms "Michael acceptor" and "Michael-type acceptor" are also used herein synonymously. Polymers suitable for use in the present invention are also discussed above and include, for example, PEG, POG, and PVA.

"Michael acceptors" are functional groups susceptible to Michael addition. "Michael addition" involves a nucleophilic attack on an electrophilic center which is adjacent to a pi system, having an electronegative atom. Examples of pi systems having an electronegative atom include sulfoxide, sulfonyl, carbonyl and heterocyclic aromatics. The nucleophile adds to the electrophilic center.

Michael acceptors can be represented by the formula:



where E is an electronegative atom. Addition takes place at the 4 position to form the following:



where N_u represents the nucleophile now bonded to the atom at position 4. Michael acceptor functional groups include, but are not limited to, maleimide and vinyl sulfone. The activated polymer from which a dumbbell is formed can, but need not, contain a vinyl sulfone species of Michael acceptor.

Activated polymers of the present invention include PEG having two or more Michael acceptor groups, including for example, PEG-*bis*-vinyl sulfone and PEG-*bis*-maleimide. PEG-*bis*-vinyl sulfone has been prepared as described in Examples 7. PEG-*bis*-maleimide has been prepared as described in PCT Publication No. WO 92/16221, incorporated herein by reference.

At least one of R_1 and R_2 is biologically active prior to coupling to X or to X-R. "Biologically active" has the same definition recited above. As stated above, biologically active molecules include, but are not limited to, binding proteins and targeting groups. Both R_1 and R_2 can be biologically active but need not be. In some cases, if R_1 and R_2 have an affinity for the same ligand, the dumbbell can have a greater affinity for that ligand than either R_1 or R_2 alone. Published PCT Publication No. WO 92/16221 shows that the homodumbbell containing two molecules of 30kDa TNFbp linked by a PEG polymer is better at inhibiting cytotoxicity of TNFs in in-vitro assays than the 30kDa molecule alone. In certain cases, R_1 can be a molecule which directs the compound R_1-X-R_2 to a certain location in a biological system and R2 can have an affinity for a ligand in that location. Alternatively, only one of R_1 and R_2 can be biologically active in the compound R_1-X-

R_2. The nonbiologically-active group can be a surface or any other biologically-inert molecule or compound.

In the present invention, the biologically active R group has a reactive thiol moiety. The biologically active R group can be a synthetic molecule. As used herein, the term "synthetic molecule" means a molecule to which a reactive thiol moiety has been added. Synthetic molecules include, for example, muteins containing a non-native cysteine. The thiol moiety reacts with a Michael-type acceptor of the polymer to form a covalent bond.

After formation of this covalent bond, the biologically-active molecule retains its biological activity. The R group "retains its biological activity" within the meaning of the invention if, after reaction with activated polymer, it has at least one tenth of the biological activity it had before reaction with polymer, preferably at least 40%, and more preferably at least 60%.

A general method for producing dumbbells follows:

(1) Choose an R group possessing the desired biological activity, for example, a protein such as tumor necrosis factor binding protein (TNFbp).

(2) Measure activity using the relevant bioassay.

(3) Determine the number of free sulfhydryl groups, for example, cysteine residues not involved in disulfide bonding, using generally known methods in the art. One such method is described in Allen, G., "Sequencing of proteins and peptides," pp. 153-54, in Laboratory Techniques in Biochemistry and Molecular Biology, Work, T.S., and Burdon, R.H., eds. (1972). If there are no free cysteines, proceed to step 4(a). If there is one free cysteine, or only one accessible to the PEGylation reagent, proceed to the reaction step in 4(c). If the protein has more than one free cysteine, go to step 5.

(4) When R is polypeptide and no free cysteines exist:

(a) Create a mutein by inserting a cysteine or replacing a non-cysteine residue with a cysteine. Useful mutation sites include the N or C terminal ends of the protein, glycosylation sites, or lysine residues. Muteins can be routinely made,

as stated above, by chemical synthesis or recombinant technology. Alternatively, chemically add a thiol moiety.

(b) Measure activity and compare that activity with the activity measured in step 2.

(c) If the mutein retains the activity measured in step 2, react the mutein with a polymer, such as PEG, having a single sulfhydryl-preferred reactive group. If the mutein bonds to the mono-reactive PEG (becomes PEGylated), measure activity and compare that activity with the activity measured in step 2. If the PEGylated mutein retains the activity measured in step 2, react the unPEGylated mutein with a PEG having two thiol-specific Michael Acceptors, such as *bis*-maleimide, to create dumbbell molecules. Repeat the bioassay to confirm that the dumbbells retain biological activity.

If one skilled in the art desires that R_1 and R_2 be different, the *bis*-reactive polymeric group can be reacted in series with R_1 and then R_2. Prior to reacting polymer with R_1, one of the two functional groups of the polymer is blocked or protected by means well known in the chemical arts to form a protected group on X. See, for example, Greene, T.W. et al., Protective Groups in Organic Synthesis, John Wiley and Sons, Inc. (1991), incorporated herein by reference. In this context, "protected" means the functional group is not available for reaction. When X having a protected group is reacted with R_1, R_1-X, and not R_1-X-R_1, is formed. After R_1-X is formed, the blocking or protecting group is removed prior to reaction with R_2. "Deprotected" means the protective group is removed or the functional group is otherwise made available for reaction.

Alternatively, heterodumbbells can be formed by reacting R_1 with an excess of the *bis*-activated polymer to force R_1-X formation. After reaction, R_1-X is separated from the reaction mixture using chromatographic techniques well known in the art, including, for example, ion exchange chromatography. R_1-X is then reacted with R_2 to form R_1-X-R_2.

(d) If the mutein created in step 4(a) or the PEGylated mutein formed in step 4(c) does not substantially retain biological activity, start with the native protein,

create a different mutein, and repeat steps 4(b) and 4(c). In addition, the length or molecular weight of the polymer X can be changed to optimize or confer biological activity.

(5) For proteins with more than one free cysteine, monoPEGylate, bioassay, and react with the bifunctional PEGylation reagent. If higher-ordered structures are formed, i.e. more than two proteins are PEG-linked, separate the dumbbells via chromatographic methods known in the art. Where such separation is undesirable for any reason, delete or replace a free cysteine with another amino acid and proceed to step 4 (b).

(6) For non-protein biologically-active R groups, exploit free sulfhydryl groups for attachment to the polymer X. Add free sulfhydryl groups to the molecule if necessary or desirable.

One skilled in the art might choose to modify, add or delete certain steps. For example, one might choose to react active proteins with a bifunctional-PEG and skip the monoPEGylation step.

Several dumbbell molecules of the present invention have been prepared. Published PCT Application No. WO 92/16221, which is incorporated herein by reference, sets forth the preparation of the following dumbbells prepared using *bis*-maleimido-PEG: 30kDa TNF inhibitor homodumbbells, Il-2 inhibitor heterodumbbell, heterodumbbells which inhibit the classical pathway of the complement system, and IL-1ra and PDGF heterodumbbells.

Pharmaceutical compositions containing many of the conjugates or compounds (collectively, the "conjugates") of the present invention can be prepared. These conjugates can be in a pharmaceutically-acceptable carrier to form the pharmaceutical compositions of the present invention. The term "pharmaceutically acceptable carrier" as used herein means a non-toxic, generally inert vehicle for the active ingredient, which does not adversely affect the ingredient or the patient to whom the composition is administered. Suitable vehicles or carriers can be found in standard pharmaceutical texts, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980), incorporated herein by reference. Such

carriers include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline. In addition, the carrier can contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation.

The pharmaceutical compositions can be prepared by methods known in the art, including, by way of an example, the simple mixing of reagents. Those skilled in the art will know that the choice of the pharmaceutical carrier and the appropriate preparation of the composition depend on the intended use and mode of administration.

In one embodiment, it is envisioned that the carrier and the conjugate constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier can be either aqueous or non-aqueous in nature. In addition, the carrier can contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier can contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the conjugate. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations can be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing the conjugates are stored and administered at or near physiological pH. It is presently believed that administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The manner of administering the formulations containing the conjugates for systemic delivery can be via subcutaneous, intramuscular, intravenous, oral, intranasal, or vaginal or rectal suppository. Preferably the manner of administration of the formulations containing the conjugates for local delivery is via intraarticular, intratracheal, or instillation or inhalations to the respiratory tract. In addition it may be desirable to administer the conjugates to specified portions of the alimentary canal either by oral administration of the conjugates in an appropriate formulation or device.

In another suitable mode for the treatment of osteoporosis and other bone loss diseases, for example, an initial intravenous bolus injection of TNF inhibitor conjugate and IL-1 inhibitor conjugate is administered followed by a continuous intravenous infusion of TNF inhibitor conjugate and IL-1 inhibitor conjugate. For oral administration, the conjugate is encapsulated. The encapsulated conjugate can be formulated with or without pharmaceutically-acceptable carriers customarily used in the compounding of solid dosage forms. Preferably, the capsule is designed so that the active portion of the formulation is released at that point in the gastro-intestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients can be included to facilitate absorption of the conjugate. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, route of administration and the age, sex and medical condition of the pateint. In certain embodiments, the dosage and administration is designed to create a preselected concentration range of the conjugate in the patient's blood stream. For example, it is believed that the maintenance of circulating concentrations of TNF inhibitor and IL-1 inhibitor of less than 0.01 ng per mL of plasma may not be an effective composition, while the prolonged maintenance of circulating levels in excess of 10 μg per mL may have undesirable side effects. Further refinement of the

calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data.

It should be noted that the conjugate formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

The following examples are illustrative of the invention and are not intended as limitations.

EXAMPLE 1: Synthesis

The reaction steps can be illustrated structurally as follows:

(1) $PEG\text{-}OH + CH_3SO_2Cl \rightarrow PEG\text{-}OSO_2CH_3$

(2) $PEG\text{-}OSO_2CH_3 + HSCH_2CH_2OH \rightarrow PEG\text{-}SCH_2CH_2OH$

(3) $PEG\text{-}SCH_2CH_2OH + H_2O_2 \rightarrow PEG\text{-}SO_2CH_2CH_2OH$

(4) $PEG\text{-}SO_2CH_2CH_2OH + SOCl_2 \rightarrow PEG\text{-}SO_2CH_2CH_2Cl$

(5) $PEG\text{-}SO_2CH_2CH_2Cl + NaOH \rightarrow PEG\text{-}SO_2\text{-}CH{=}CH_2 + HCl$

Each of the above reactions is described in detail below:

Reaction 1. Reaction 1 represents the preparation of the methane sulfonyl ester of polyethylene glycol, which can also be referred to as the methanesulfonate or mesylate of polyethylene glycol. The tosylate and the halides can be prepared by similar procedures, which are believed to be apparent to the skilled artisan.

To prepare the mesylate, twenty-five grams of PEG of molecular weight 3400 was dried by azeotropic distillation in 150 mL of toluene. Approximately half of the toluene was distilled off in drying the PEG. Forty mL of dry dichloromethane was added to the toluene and PEG solution, followed by cooling in an ice bath. To the cooled solution was added 1.23 mL of distilled methanesulfonyl chloride, which is

an equivalent weight of 1.6 with respect to PEG hydroxyl groups, and 2.66 mL of dry triethylamine, which is an equivalent weight of 1.3 with respect to PEG hydroxyl groups. "Equivalent weight" as used above can be thought of as "combining weight" and refers to the weight of a compound that will react with an equivalent weight of PEG hydroxyl groups.

The reaction was permitted to run overnight during which time it warmed to room temperature. Triethylammonium hydrochloride precipitated and the precipitate was removed by filtration. Thereafter, the volume was reduced by rotary evaporation to 20 mL. The mesylate was precipitated by addition to 100 mL of cold dry ethyl ether. Nuclear magnetic resonance (NMR) analysis showed 100% conversion of hydroxyl groups to mesylate groups.

Reaction 2. Reaction 2 represents the formation of polyethylene glycol mercaptoethanol by reaction of the mesylate with mercaptoethanol. The reaction causes the methanesulfonate radical to be displaced from the PEG. The sulfur in the mercaptoethanol radical is attached directly to the carbon in the carbon-carbon backbone of the PEG.

Twenty grams of the mesylate from reaction 1 was dissolved in 150 mL of distilled water. The solution of mesylate and water was cooled by immersion in an ice bath. To the cooled solution was added 2.37 mL of mercaptoethanol, which is 3 equivalent weights with respect to PEG hydroxyl groups. Also added was 16.86 mL of 2N NaOH base. The reaction was refluxed for 3 hours, which means that the vapors rising from the heated reaction were continuously condensed and allowed to flow back into the reaction.

The polyethylene glycol mercaptoethanol product was extracted three times with dichloromethane using approximately 25 mL of dichloromethane each time. The organic fractions were collected and dried over anhydrous magnesium sulfate. The volume was reduced to 20 mL and the product was precipitated by addition to 150 mL of cold dry ether.

NMR analysis in d_6-DMSO (dimethyl sulfoxide) gave the following peaks for PEG-SCH_2CH_2OH: 2.57 ppm, triplet, -CH_2-S-; 2.65 ppm, triplet, -S-CH_2-; 3.5 ppm,

backbone singlet; and 4.76 ppm, triplet, -OH. Integration of the peak for -S-CH_2- indicated 100% substitution.

Reaction 3. Reaction 3 represents peroxide oxidation of the polyethylene glycol mercaptoethanol product to convert the sulfur, S, to sulfone, SO_2. PEG-β-hydroxysulfone is produced.

Twenty grams of PEG-SCH_2CH_2OH was dissolved in 30 mL of 0.123M tungstic acid solution and cooled in an ice bath. The tungstic acid solution was prepared by dissolving the acid in sodium hydroxide solution of pH 11.5 and then adjusting the pH to 5.6 with glacial acetic acid. Twenty mL of distilled water and 2.88 mL of 30% hydrogen peroxide, which has an equivalent weight of 2.5 with respect to hydroxyl groups, was added to the solution of tungstic acid and polyethylene glycol mercaptoethanol and the reaction was permitted to warm overnight to room temperature.

The oxidized product was extracted three times with dichloromethane using 25 mL of dichloromethane each time. The collected organic fractions were washed with dilute aqueous sodium bicarbonate and dried with anhydrous magnesium sulfate. The volume was reduced to 20 mL. The PEG-β-hydroxysulfone product was precipitated by addition to cold dry ethyl ether.

NMR analysis in d_6-DMSO gave the following peaks for PEG-SCH_2CH_2OH: 3.25 ppm, triplet, -CH_2-SO_2-; 3.37 ppm, triplet, -SO_2-CH_2-; 3.50 ppm, backbone; 3.77 ppm, triplet, -CH_2OH; 5.04 ppm, triplet, -OH. The hydroxyl peak at 5.04 ppm indicated 85% substitution. However, the peak at 3.37 ppm for -SO_2-CH_2- indicated 100% substitution and is considered to be more reliable.

Reaction 4. Reaction 4 represents the final step in synthesis, isolation, and characterization of polyethylene glycol chloroethyl sulfone.

To synthesize the product, twenty grams of PEG-$SO_2CH_2CH_2OH$, PEG-β-hydroxysulfone, was dissolved in 100 mL of freshly distilled thionyl chloride and the solution was refluxed overnight. The thionyl chloride had been distilled over quinoline. Excess thionyl chloride was removed by distillation. Fifty mL of toluene and 50 mL of dichloromethane were added and removed by distillation.

To isolate the product, the PEG chloroethyl sulfone was dissolved in 20 mL of dichloromethane and precipitated by addition to 100 mL of cold dry ethyl ether. The precipitate was recrystallized from 50 mL of ethyl acetate to isolate the product.

Nuclear magnetic resonance was used to characterize the product. NMR analysis of PEG-$SO_2CH_2CH_2Cl$ in d_6-DMSO gave the following peaks: 3.50 ppm, backbone; 3.64 ppm, triplet, -CH_2SO_2-; 3.80 ppm, triplet, -SO_2-CH_2-. A small hydroxyl impurity triplet appeared at 3.94 ppm. Calculation of the percentage substitution was difficult for this spectrum because of the proximity of the important peaks to the very large backbone peak.

Reaction 5. Reaction 5 represents conversion of polyethylene glycol chloroethyl sulfone from reaction step 4 to polyethylene glycol vinyl sulfone and isolation and characterization of the vinyl sulfone product.

The PEG vinyl sulfone was readily prepared by dissolving solid PEG chloroethyl sulfone in dichloromethane solvent followed by addition of two equivalents of NaOH base. The solution was filtered to remove the base and the solvent was evaporated to isolate the final product PEG-SO_2-CH=CH_2, PEG vinyl sulfone.

The PEG vinyl sulfone was characterized by NMR analysis in d_6-DMSO dimethyl sulfoxide. NMR analysis showed the following peaks: 3.50 ppm, backbone; 3.73 ppm, triplet, -CH_2-SO_2-; 6.21 ppm, triplet, =CH_2; 6.97 ppm, doublet of doublets, -SO_2-CH-. The 6.97 ppm peak for -SO_2-CH- indicated 84% substitution. The 6.21 ppm peak for =CH_2 indicated 94% substitution. Titration with mercaptoethanol and 2,2'-dithiodipyridine indicated 95% substitution.

EXAMPLE 2: Thiol-selective Reactivity

Example 2 shows that PEG vinyl sulfone and its precursor PEG chloroethyl sulfone are significantly more reactive with thiol groups (-SH) than with amino groups (-NH_2) or imino groups (-NH-). Compounds containing thiol groups are organic compounds that resemble alcohols, which contain the hydroxyl group -OH, except that in thiols, the oxygen of the hydroxyl group is replaced by sulfur. Thiols

sometimes are also called sulfhydryls or mercaptans. PEG vinyl sulfone contains the vinyl sulfone group $-SO_2-CH=CH_2$. PEG chloroethyl sulfone contains the chloroethyl sulfone group $-SO_2CH_2CH_2Cl$.

Selectivity for thiols is important in protein modification because it means that cysteine units (containing -SH) will be modified in preference to lysine units (containing $-NH_2$) and histidine units (containing -NH-). The selectivity of PEG vinyl sulfone for thiols means that PEG can be selectively attached to cysteine units, thus preserving protein activity for specific proteins and controlling the number of PEG molecules attached to the protein.

The relative reactivity of PEG vinyl sulfone with thiol and amino groups was determined by measuring the rates of reaction of PEG vinyl sulfone with N-α-acetyl lysine methyl ester and with mercaptoethanol. N-α-acetyl lysine methyl ester is a lysine model containing an amino group and is abbreviated Lys-NH_2. Mercaptoethanol serves as a cysteine model containing a thiol group and is abbreviated Cys-SH. Relative reactivity of PEG chloroethyl sulfone was similarly determined. This molecule may serve as a "protected" form of the vinyl sulfone since it is stable in acid but converts to PEG vinyl sulfone upon addition of base.

Reactivity for PEG vinyl sulfone and for the PEG chloroethyl sulfone precursor was investigated at pH 8.0, pH 9.0, and at pH 9.5. Buffers for controlling the pH were 0.1 M phosphate at pH 8.0 and 0.1 M borate at pH 9.0 and at pH 9.5. For measurement of mercaptoethanol reactivity, 5 mM ethylenediamine tetraacetic acid (EDTA) was added to both buffers to retard conversion of thiol to disulfide.

For reaction of the PEG derivatives of the invention with Lys-NH_2, a 3 mM solution of the PEG derivative was added under stirring to a 0.3 mM Lys-NH_2 solution in the appropriate buffer for each of the three levels of basic pH. The reaction was monitored by addition of fluorescamine to the reaction solution to produce a fluorescent derivative from reaction with remaining amino groups. The monitoring step was performed by adding 50 μL of reaction to 1.95 mL of phosphate buffer of pH 8.0 followed by adding 1.0 mL of fluorescamine solution under vigorous stirring. The fluorescamine solution was 0.3 mg fluorescamine per mL of acetone.

Fluorescence was measured 10 minutes after mixing. Excitation was at wavelength 390 nm. Light emission occurred at 475 nm. No reaction was observed in 24 hours for either PEG vinyl sulfone or PEG chloroethyl sulfone at pH 8.0. At pH 9.5 the reaction was slow, but all amino groups were reacted after several days.

For reaction of the PEG vinyl sulfone and PEG chloroethyl sulfone precursor with Cys-SH, a 2 mM solution of the PEG derivative was added to a 0.2 mM solution of Cys-SH in the appropriate buffer for each of the three levels of basic pH. The reaction was monitored by adding 4-dithiopyridine to the reaction solution. The 4-dithiopyridine compound reacts with Cys-SH to produce 4-thiopyridone, which absorbs ultraviolet light.

The monitoring step was performed by adding 50μL of reaction mixture to 0.95 mL of 0.1 M phosphate buffer at pH 8.0 and containing 5 mM EDTA, followed by adding one mL of 2 mM 4-dithiopyridine in the same buffer.

Absorbance of 4-thiopyridone was measured at 324 nm. Both PEG vinyl sulfone and PEG chloroethyl sulfone showed reactivity toward Cys-SH, with PEG vinyl sulfone showing greater reactivity. At pH 9.0 the reaction is over within two minutes using the vinyl sulfone and within 15 minutes using the chloroethyl sulfone. However, these reactions were too fast for determination of accurate rate constants. At pH 8.0 the reactions were slower, but still complete in one hour for vinyl sulfone and in three hours for the chloroethyl sulfone. The conversion of chloroethyl sulfone to vinyl sulfone is significantly slower than the reaction of vinyl sulfone with Cys-SH. Thus the rate of reaction for chloroethyl sulfone with Cys-SH appears to be dependent on the rate of conversion of chloroethyl sulfone to vinyl sulfone. Nevertheless, these reaction rates were still much faster than for the reaction with Lys-NH_2.

The above kinetic studies demonstrate the following points. PEG vinyl sulfone is much more reactive with thiol groups than with amino groups, indicating that attachment of PEG vinyl sulfone to a protein containing both cysteine and lysine groups proceeds primarily by reaction with cysteine. Since reactivity with amino groups is similar to imino groups, then reactivity of histidine subunits will also be much lower than reactivity with cysteine subunits. Also, selectivity toward thiol

groups is accentuated at lower pH values for PEG chloroethyl sulfone and PEG vinyl sulfone, although the reactions of PEG chloroethyl sulfone are somewhat slower.

The utility of many PEG derivatives is limited because they react rapidly with water, thus interfering with attempts to attach the derivative to molecules and surfaces under aqueous conditions. The following Example 3 shows that PEG vinyl sulfone and PEG chloroethyl sulfone are stable in water.

EXAMPLE 3: Hydrolytic Stability

PEG vinyl sulfone was dissolved in heavy water, D_20 deuterium oxide, and monitored by NMR. Reaction did not occur. A solution of PEG chloroethyl sulfone produced PEG vinyl sulfone in heavy water that was buffered with borate to pH 9.0. Monitoring with NMR showed that PEG vinyl sulfone, once produced, was stable for three days in heavy water.

PEG chloroethyl sulfone is stable in water until solution becomes basic, at which time it is converted into vinyl sulfone. Conversion to vinyl sulfone has been demonstrated by dissolving PEG chloroethyl sulfone in water at pH 7 and in borate buffer at pH 9. The PEG derivative is extracted into methylene chloride. Removal of methylene chloride followed by NMR analysis showed that PEG chloroethyl sulfone is stable at a neutral pH of 7.0, and reacts with base to produce PEG vinyl sulfone.

Vinyl sulfone is stable for several days in water, even at basic pH. Extensive hydrolytic stability and thiol-specific reactivity of PEG vinyl sulfone means that PEG vinyl sulfone and its precursor are useful for modification of molecules and surfaces under aqueous conditions, as shown in the following Example 4.

EXAMPLE 4: Conjugation to BSA

Protein modification was demonstrated by attachment of the PEG derivative to bovine serum albumin (BSA) by two different methods. BSA is a protein. Native unmodified BSA contains cystine groups which do not contain thiol groups. The cystine units are tied up as disulfide linkages, S-S.

In the first method, m-PEG (monomethoxy-PEG) vinyl sulfone of molecular weight 5,000 was reacted with unmodified BSA for 24 hours in a 0.1 M borate buffer at pH 9.5 at room temperature. The solution contained 1 mg of BSA and 1 mg of m-PEG vinyl sulfone, of molecular weight 5,000, per mL of solution. The results from the Example 2 model compounds had indicated that lysine subunits (and possibly histidine subunits) would be modified under these relatively basic conditions and in the absence of free thiol groups available for reaction.

Attachment to lysine subunits was demonstrated in two ways. First, size exclusion chromatography showed that the molecular weight of the protein had increased by approximately 50%, thus indicating attachment of approximately 10 PEGs to the protein. Second, fluorescamine analysis showed that the number of lysine groups in the BSA molecule had been reduced by approximately ten.

In the second method, the BSA was treated with tributylphosphine to reduce the disulfide S-S bonds to thiol groups, -SH, which are available for reaction. The modified BSA was then treated with PEG chloroethyl sulfone at pH 8.0 in a 0.1 M phosphate buffer at room temperature for 1 hour. The solution contained 1 mg of modified BSA and 1 mg of m-PEG chloroethyl sulfone of molecular weight 5,000 per mL of solution. The results showed that lysine groups were unreactive under these conditions. However, thiol groups were reactive.

Attachment of the PEG to the protein was demonstrated by size exclusion chromatography, which showed an increase in the molecular weight of the protein by about 25%. Fluorescamine analysis indicated no change in number of lysine subunits in the protein, thus confirming that PEG attachment did not take place on lysine subunits. Substitution on thiol groups was thereby confirmed.

EXAMPLE 5: Synthesis of vinyl sulfone NHS-ester heterobifunctional PEG (3,400) reagent.

Briefly, PEG(3,400)-ω-vinyl sulfone-α-priopionic acid, succinimidyl ester was synthesized in several steps. First, the ethyl ester of PEG(3,400)-ω-hydroxy-α-propionic acid was synthesized. Second, the ethyl ester was converted to the ω-mesylate derivative. Third, the mesylate was used to prepare the ω-thioethanol derivative. Fourth, the thioethanol derivative was converted to the ω-hydroxysulfone. Fifth, the hyroxysulfone was converted to the ω-vinyl sulfone. The latter α-ethyl ester was converted to the α-propionic acid in a sixth step. Finally, the propionic acid group was converted to the succinimidyl ester. The detailed synthesis is set forth below.

Step 1. 15.0 grams of PEG(3,400)-ω-hydroxy-α-propionic acid, 75 mL anyhydrous ethyl alcohol, and 3 mL sulfuric acid were heated to reflux for 1 hour. After cooling to room temperature, 50 mL water was added to the reaction mixture and sodium bicarbonate was used to adjust pH to 7. Ethyl alcohol was distilled off under reduced pressure using a rotoevaporator at 55°C for one-half hour. The reaction product was extracted with 60, 50 and 40 mL dichloromethane. The extract was dried with anhydrous magnesium sulfate, concentrated to 50 mL, and added to 400 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield of the ethyl ester was 13.1 grams. NMR analysis showed 49% propionic acid, ethyl ester groups and 51% PEG-OH groups.

Step 2. A mixture of 13.0 grams (0.0038 mol) of the ethyl ester derivative formed in step 1, 100 mL toluene, and 2.0 grams BHT was azeotropically dried during heating to reflux. Next, 15 mL dry dichloromethane, 0.60 mL (0.0043 mol, 1.15 fold excess) triethylamine and 0.31 mL (0.0040 mol, 1.07 fold excess) mesyl chloride were added at 5°C and the mixture was stirred overnight at room temperature under a nitrogen atmosphere. 2 mL anhydrous ethyl alcohol was added and the mixture was stirred for 15 minutes. The mixture was then filtered and about 70 mL of solvents were distilled off under reduced pressure to yield a toluene solution of PEG-ω-mesylate-α-propionic acid ethyl ester.

Step 3. The following were added to about 40 mL (0.00375 mol) of the PEG-ω-mesylate-α-propionic acid ethyl ester solution obtained in step 2: 150 mL of anyhydrous ethyl alcohol, 1.79 mL (0.0139 mol, 3.69 fold excess) mercaptoethanol and 0.45 grams (0.0011 mol, 3.0 fold excess) sodium hydroxide dissolved in 20 mL anhydrous ethyl alcohol. The mixture was heated 3 hours at 58-62°C under a nitrogen atmosphere. After cooling to room temperature, acetic acid was used to adjust the pH to about 6.5 and 140 mL of ethyl alcohol was distilled off under reduced pressure using a rotoevaporator, at 55°C for 40 minutes. After distillation, 50 mL dichloromethane was added to the residue. The resulting solution was washed with distilled water and dried with anhydrous magnesium sulfate. The solution was then concentrated to 30 mL and added to 350 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield of the thioethanol derivative was 11.5 grams. NMR analysis showed 52% thioethanol groups, 35% propionic acid, ethyl ester groups and 13% PEG-OH moieties.

Step 4. Next, a solution of 11.5 grams PEG-ω-thioethanol-α-propionic acid, ethyl ester in 12 mL distilled water was prepared. A tungstic acid solution was also prepared as follows: 0.14 grams of tungstic acid, 12.0 mL distilled water and 0.05 grams sodium hydroxide dissolved in 6.0 mL water were mixed to form a solution having a pH of 11.5. A 10% solution of NaH_2PO_4 was added to the tungstic acid solution to adjust the pH to 6.6. The 12 mL solution of ethyl ester was then added to the pH 6.6 tungstic acid solution and the pH was again adjusted to 6.6 with 0.1M NaOH. 1.1 mL of 30% hydrogen peroxide was added and the reaction mixture was stirred for 19 hours. The pH after the reaction period was 6.7. 1M NaOH was added to adjust the pH to 7.2 and the reaction mixture was stirred for 1 hour. 5 grams of sodium chloride dissolved in 45 mL distilled water was added to the reaction mixture. The reaction product was extracted 3 times with 50 mL dichloromethane. The extract was dried with magnesium sulfate as follows: 10 grams powdered magnesium sulfate was added to the extract and the magnesium sulfate was filtered away after two hours. The magnesium sulfate dried extract was concentrated to 40 mL and added to 350 mL cold diethyl ether. The precipitated product was

filtered off and dried under reduced pressure. The yield was 9.7 grams and contained 50% hydroxysulfone groups, 39% propionic acid, ethyl ester groups and 11% PEG-OH groups as determined by NMR.

Step 5. To a mixture of: 9.6 grams (0.00271 mol) of the PEG-ω-hydroxysulfone-α-propionic acid, ethyl ester synthesized in step 4, 50 mL dichloromethane and 0.01 grams (0.1 wt % per PEG) BHT stirred at room temperature under a nitrogen atmosphere was added 3.00 mL (0.0215 mol, 3.97 fold excess) triethylamine and 0.80 mL (0.010 mol, 3.81 fold excess) mesyl chloride. The reaction mixture was stirred for 15 minutes, filtered, and diluted with 150 mL dichloromethane. The resulting mixture was then washed with 25 mL 1M HCl, 25 mL 10% NaCl and 25 mL water. A small amount of Na_2HPO_4 was added to adjust the pH of the water layer to 7. The reaction mixture was then dried with magnesium sulfate and concentrated to 40 mL. The obtained solution was added to 400 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure to yield 9.1 grams. NMR analysis showed the following functionalities: 43% vinyl sulfone, 16% mesylate, and 35% propionic acid, ethyl ester.

Step 6. To a solution of 9.0 grams of the PEG-ω-vinyl sulfone-α propionic acid, ethyl ester derivative in 50 mL distilled water, 1.0M NaOH was added to adjust the pH to 12.0 and the solution was stirred 1.5 hours keeping the pH between 11.9 and 12.1 by periodic addition of 1.0M NaOH. Next, the pH was adjusted to 3.0 with oxalic acid, 5 grams of NaCl was added to the solution, and the reaction product was extracted 3 times with 50 mL dichloromethane. The extract was dried with anhydrous magnesium sulfate, concentrated to 30 mL and added to 350 mL cold diethyl ether. The precipitate was filtered off and dried under reduced pressure. The yield was 6.8 grams. Functional groups identified by NMR analysis were: vinyl sulfone 40%, propionic acid 29%, propionic acid, ethyl ester 4%, and 17% mesylate. The precipitate was purified by ion-exchange chromatography over a DEAE Sepharose FF column. The yield after purification was 3.2 grams and NMR analysis showed 50% propionic acid groups, 38% vinyl sulfone groups, and 8% mesylate groups.

Step 7. A mixture of 3.0 grams PEG-ω-vinyl sulfone-α-propionic acid, 0.12 grams N-hydroxysuccinimide, 0.21 grams DCC (dicyclohexylcarbodiimide) in 20 mL dichloromethane was stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was then filtered and added to 250 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure to yield 2.90 grams. NMR showed the following groups: succinimide 50%, 38% vinyl sulfone, 10% mesylate, and 2% hydroxysulfone.

EXAMPLE 6: Synthesis of maleimide, NHS-ester heterobifunctional PEG (3,400) reagent.

The maleimide, NHS-ester PEG reagent was synthesized in two steps. In the first step, maleimido-PEG-OH was synthesized. Specifically, 0. 130 grams maleimido succinimidyl propionate were dissolved in 5 mL dry dichloromethane and cooled to 0°C. Next, 0.5 grams PEG-monoamine, prepared as described below, was added and then 2 drops of triethylamine. After 2 hours at room temperature, TLC indicated that the reaction was complete. TLC was conducted using n-BUOH-ACOH-H_2O at a ratio of 4:1:1. The reaction mixture was evaporated to dryness and the residue dissolved in 15 mL distilled water. The pH of the solution was adjusted to 3 using 15 mL 0.5M HCl and extracted with 10 mL CH_2Cl_2. The organic layer was dried with magnesium sulfate, filtered, concentrated to 15 mL, and poured into 75 mL cold ether. The precipitate was filtered and dried in vacuo. The yield was 0.300 grams. NMR analysis showed 77% maleimide groups and 100% PEG-OH.

In the second step, the maleimido-PEG-OH was converted to the maleimide-PEG-NHS-ester. A mixture of 2 mL CH_2Cl_2, 0.05 mL pyridine (1 equivalent) 1 mL acetonitrile and 0.266 grams maleimido-PEG-OH was stirred at room temperature under nitrogen. To this mixture, 0.070 grams (2.5 equivalents) N,N-disuccinimidyl carbonate was added and the reaction left overnight. The reaction mixture was then poured into approximately 50 mL cold ether, filtered and dried in vacuo. The NMR showed impurities and the product was precipitated a second time with a final yield of 0.230 grams.

The PEG-monoamine used in the first step above was prepared in three steps as follows. First, the PEG-mesylate derivative was formed. From the mesylate, the amine was formed. Finally, the monoamine was separated from the underivatized PEG and the diamine.

Step 1 PEG-3,400 (120 grams, 0.07164 equivalents of OH) was dissolved in 580 mL toluene, azeotropically dried, and then 90 mL dichloromethane, 1.80 mL triethylamine (0.01291 mol) and 0.83 mL mesyl chloride (0.01072 mol) were added. After overnight reaction at room temperature, 90 mL of solvents were distilled off from the reaction mixture under reduced pressure, the mixture was filtered and then 500 mL toluene was distilled off under reduced pressure. The residue was added to 800 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield was 118 grams and the substitution was 15%.

Step 2 118 grams of the mesylate formed in step 1 and 80 grams ammonium chloride were dissolved in 1600 mL concentrated aqueous NH_4OH and stirred at room temperature for 44 hours. The reaction product was extracted with 600, 400, and then 200 mL dichloromethane. The extract was washed with 170 mL 2% KOH and 170 mL water, dried with magnesium sulfate, concentrated to 200 mL and added to 800 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield was 106 grams and the substitution was 15.6%.

Step 3 45 grams of the amine formed in step 2 was dissolved in 9 L water and loaded onto SP-Sepharose FF (300 mL of gel equilibrated with 1000 mL citric acid-lithium citrate buffer, 0.4%, pH 3.0, and then washed with water). SP-Sepharose FF is available from Pharmacia, Uppsala, Sweden. The underivatized PEG was washed off the column with water. Next, PEG monoamine was eluted with 800 mL 20 mM NaCl. The pH of the eluate was adjusted to 11 with 1M NaOH and the PEG monoamine was extracted with dichloromethane, dried with magnesium sulfate, and the solvent was distilled off. The yield was 9 grams.

EXAMPLE 7: SYNTHESIS OF PEG-α,ω-*bis*-vinyl sulfone.

The synthesis of 3,400 and 20,000 kDa PEG *bis*-vinyl sulfone was conducted using PEG diol and the general method set forth above. PEG diol was purchased from Fluka Chemical Corporation (Ronkonkoma, New York) or from Nippon Oil and Fat (Tokyo, Japan).

EXAMPLE 8: PEGYLATION of IL-1ra using PEG-20,000 -α,ω-*bis*-vinyl sulfone.

The IL-1ra c84 mutein was prepared as set forth in published PCT Application WO 92/16221, incorporated herein by reference. Conjugation of the c84 mutein or the native (wild-type) IL-1ra using PEG-α,ω-*bis*-vinyl sulfone (3,400 or 20,000 kDa) was conducted at 25°C in citrate buffer, pH 6.75-7.5, in 1 mL tubes, varying PEG and protein concentrations. At a protein concentration of 30 mg/mL, good conversion to the dumbbell molecule was obtained within 18 hours. At a protein concentration of 0.94 mg/mL, mostly monoadducts were obtained. The dumbbell species was preferentially formed at a protein concentration of 100 mg/mL with 0.03 equivalents PEG. The dumbbell can be purified using chromatographic techniques set forth in PCT Publication Publication No. WO 92/16221, incorporated herein by reference.

In other experiments 0.1 M Tris-HCl buffer, pH 8.5, containing 30 mg/mL of the wild-type IL-1ra was treated with a 0.53 molar equivalent of the 20kDa PEG-*bis*-vinyl sulfone at 25°C for 18 hours. SDS PAGE analysis showed conversion to both dumbbell and the monoadduct. At a protein concentration of 3.1 mg/mL with 1 molar equivalent of PEG reagent, only the monoadduct was observed.

In general, the c84 mutein reacts more readily with the PEG reagent than the wild-type molecule.

EXAMPLE 9. Bioactivity of IL-1ra dumbbell.

The c84 dumbbell generated above was analyzed for its receptor binding affinity compared to that of unPEGylated recombinant IL-1ra on murine EL-4 cells using the

assay set forth in PCT Application Publication No. WO 92/16221, incorporated herein by reference. The results showed similar binding affinities between the two molecules.

EXAMPLE 10: PEGYLATION of TNFbp c105 mutein with PEG-20,000-α, ω-*bis*-vinyl sulfone.

The c105 mutein of TNFbp was prepared as set forth in published PCT Publication WO 92/16221, incorporated herein by reference. Alternatively, the c105 mutein was prepared as follows.

E. Coli cells expressing the c105 mutein were harvested by centifugation. The cell sludge was adjusted to approximately 40% wet weight solids by the addition of purified water. The mixture was then further diluted with an equal volume of breaking buffer (50 mM Tromethamine, 4 mM EDTA, pH 7.2) to give a suspension with approximately 20% wet weight solids. The cell sludge was passed five times through a high pressure homogenizer operating at approximately 8,000 psi to produce the cell homogenate. The homogenate was cooled to less than or equal to 10°C prior to each pass through the homogenizer. The homogenate was centrifuged and the solids fraction containing the c105 was retained. The solids were diluted and centrifuged again to give washed inclusion bodies.

The washed inclusion bodies were then dissolved by addition of 8 M urea and 150 mM cysteine in 50 mM TRIS, pH 9.5 This mixture was allowed to stir for two hours at room temperature prior to refolding. Under these conditions, the c105 mutein was denatured and reduced.

The reduced denatured c105 mutein was refolded by dilution with 1.1 M urea, 50 mM Tris to give a final refold solution comprised of 200ug/mL c105 mutein, 1.5 M urea, 7.5 mM cysteine, 50 mM Tris, pH 9.7. The refold mixture was held at 6-10°C for two days. Refold efficiency was monitored by reverse phase HPLC and cation exchange HPLC.

The refold mixture was then brought to pH 5.0 by addition of acetic acid and HCl. The refold mixture was loaded onto a cation exchange column (S-Sepharose big

bead resin) previously equilibrated in 25 mM sodium acetate, 65 mM NaCl, pH 5 at 4°C. After loading, the column was washed with the same equilibration buffer. The column was eluted with a gradient from 65 to 350 mM NaCl in 25 mM sodium acetate, pH 5. The c105 mutein eluted at about 200 mM NaCl and was collected in one pool.

The collected pool containing the c105 mutein was diluted with 1.5 volumes of 5 M NaCl, 40 mM sodium phosphate, adjusted to pH 6, and loaded onto a hydrophobic interaction column (Toyo Butyl 650 M column), previously equilibrated in 3 M NaCl, 20 mM sodium phosphate, pH 6. At the end of the load, the column was washed with equilibration buffer. The c105 mutein was eluted using a linear eight column volume decreasing salt gradient running from 3 M to 1 M NaCl, in 20 mM sodium phosphate, at pH 6. The c105 mutein was collected in one pool. The pool was then concentrated to approximately 3 g/L c105 mutein and then diafilted against 20 mM sodium phosphate, pH 6.0 until the final conductivity was less than 4 mmho (approximately six volumes).

The diafiltered pool was loaded onto a SP-Sepharose high performance column equilibrated in 20 mM sodium phosphate, pH 6.0. After loading, the column was washed with additional equilibration buffer and eluted with a combination pH/salt gradient from 20 mM sodium phosphate, 50 mM NaCl, pH 6.0 to 20 mM sodium phosphate, 50 mM NaCl, pH 6.5. The c105 mutein eluted in the later half of the gradient at about 35 mM NaCl. The c105 mutein can be stored frozen at this point.

The c105 mutein was reacted with the PEGylation reagent at molar ratios of PEG reagent to protein of 1:1, 2:1, 4:1, 1:2 and 0:1 (control). The reaction was carried out in 20 mM phosphate/ 20mM acetate buffer at pH 7.5 for 15 hours at 22°C. Reactions were also carried out in 50mM phosphate buffer, pH 7.5 or 8.5.

The percent conversion to the dumbbell molecule was determined by cation exchange HPLC over a MA7S column. The percent conversion ranged from approximately 40-60%. Conversion to the dumbbell molecule was optimized by adding a solution of approximately 50mg/mL of PEG reagent to the protein at a molar ratio of 0.50-0.65 PEG reagent to 1.0 of TNFbp mutein at pH 7.5 for 15 hours at

22°C. As the ratio of PEG to protein is increased, production of the monoadduct was favored. Monoadduct formation was optimized by a 5:1 ratio of PEG reagent to protein.

Conjugates were purified by chromatography over an S-Sepharose HP column. The reaction mixture was adjusted to pH 3.0-4.2 and loaded onto the column previously adjusted to the same pH. The column was washed with an equilibration buffer and the dumbbell was eluted using a liner sodium chloride gradient and a flow rate of 1.2-1.5 cm/min. The following species eluted from the column in the following order: 1) monosubstituted, 2) dumbbell, 3) unPEGylated TNFbp mutein, and 4) aggregated mutein.

EXAMPLE 11. Bioactivity of TNFbp c105 mutein dumbbell.

c105 dumbbells, whether formed from PEG-*bis* maleimide as described in PCT Application Publication No. WO 92/16221 or as described herein, were shown to be 50 to 100 fold more active than the unPEGylated 30kDa TNF inhibitor by comparison in the L929 cytotoxicity assay set forth in WO 92/16221, incorporated herein by reference.

EXAMPLE 12: Preparation of glyceryl-PEG-tris-vinyl sulfone

Glyceryl-PEG-α,β,γ-triol (10,000 kDa and 20,000 kDa) was converted to the vinyl sulfone derivative using the general method described above. Glyceryl-PEG-α,β,γ-triol was purchased from Union Carbide, Terrytown, New York. Glyceryl-PEG-α,β,γ-triol can be synthesized by ethylene oxide polymerization off of glycerol in base.

EXAMPLE 13: Synthesis of TNFbp c105 trumbbell using glyceryl-PEG-tris-vinyl sulfone

Three TNFbp c105 muteins were conjugated to PEG-tris-vinyl sulfone to yield a "trumbbell" molecule. Experiments conducted over a wide range of PEG:protein ratios showed that a particularly useful molar ratio for conversion to the trumbbell

was 0.25-0.35 PEG to 1 protein. In a typical experiment, the c105 mutein in 20mM phosphate, 20mM acetate buffer, pH 7.5 was exposed to a 0.03 molar equivalent of glyceryl-PEG-10,000-α, β, γ-triol at 25°C for 18 hours. Analysis of the latter reaction mixture by cation exchange HPLC (Bio Rad MA7S column eluting a sodium chloride gradient) indicated conversion to the trumbbell in 49% yield and bi-substitution in a 34.9% yield.

EXAMPLE 14: Synthesis of IL-1ra trumbell using glyceryl-PEG-tris-vinyl sulfone.

A solution of PEG-10,000-α,β,γ-tris-vinyl sulfone was reacted with 20 mg/mL wild-type IL-1ra in 0.1 M phosphate buffer at the following PEG/protein molar ratios: 0.10:1; 0.25:1; 0.35:1; 0.45:1; 0.55:1; 0.65:1. The reactions were incubated at 25°C for 72 hours. SDS PAGE analysis showed conversion to mono, di, and triadducted products. Optimal conversion to the triadduct was observed at a PEG/protein ratio of 0.10:1. The reaction mixture was applied to an S Sepharose high performance column and eluted with a sodium chloride gradient.

EXAMPLE 15. Synthesis of c105 TNFbp-PEG-IL-1ra heterodumbbell

A solution of wild-type IL-1ra in 0.1M phosphate buffer, pH 8.5 was reacted with 8 mg/mL PEG-20,000-*bis*-vinyl sulfone-mono-c105TNFbp adduct at the following molar ratios and concentrations of IL-1ra indicated: 55:1 (12.5 mg/mL); 85:1 (18.75 mg/mL); 100:1 (25.0mg/mL) and 150:1 (31.75 mg/mL). After 72 hours, heterodumbbell was formed as determined by SDS PAGE. Optimal conversion was observed at a ratio of 1:100 monoadduct to IL-1ra. The heterodumbbell was purified using an S Sepharose high performance column and eluting with a sodium chloride gradient.

EXAMPLE 16. Stability of PEG-vinyl sulfone polypeptide adducts

The stability of the linkage between the c105 TNFbp mutein and PEG-*bis*-vinyl sulfone was studied. Known amounts of the c105 dumbbell were incubated in PBS,

pH7.4, at 37°C for up to one week with aliquots removed at intervals for analysis by SDS PAGE. Essentially no decomposition of the c105 dumbbell was observed. At pH 10 at 37°C for 1 week, only 5-10% degradation of the conjugate was observed.

EXAMPLE 17. TNFbp c105 dumbbell inhibits actively-induced experimental allergic encephalomyelitis ("EAE").

The in vivo activity of the c105 dumbbell made with PEG-*bis*-vinyl sulfone has been demonstrated. EAE is a murine model of an autoimmune inflammatory demyelinating disease of the central nervous system that is often used as a model for human MS. AS described below, the c105 dumbbell inhibited EAE in rats.

Female Lewis rats (150-200g) were purchased from Charles River (Raleigh, NC), and housed for at least 1 week before starting experiments. They received food and water *ad libitum* and were housed in temperature and light controlled (12h/day) rooms. Within each experiment, animals were age-matched.

Active induction of EAE Rats (usually six per group) were anesthetized with 2% isoflurane + O_2 and immunized on day 0 in the footpad of the left hind limb with 0.1 mL of an emulsion containing myelin basic protein ("MBP") at one of the following doses; 0, 1, 3, 10 or 30 μg (fragment 68-84 Bachem Bioscience, PA). The MBP was dissolved in phosphate buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/mL of *Mycobacterium tuberculosis* H37Ra (Difco Lab, MI). Control rats received 0.1 mL of the PBS/CFA emulsion with no MBP in the footpad of the left hindlimb.

Clinical Scoring of EAE Evaluation of clinical disease was performed on a daily basis using a standard 0-5 scoring system. Briefly, the spectrum of rating was 0 normal, 0.5 partial loss of tail tone, 1 complete loss of tail tone, 2 dragging of one hind limb, 3 paralysis of both hind limbs, 4 morbid, and 5 death. Daily weights were recorded for individual rats and weight loss/gain was expressed relative to initial weight.

Effects of immunization with MBP Initial studies assessed the clinical severity of different doses of MBP (0.1-30 μg/ 0.1 mL) in the emulsion described above in

the rat. The 0.1 and 0.3 μg MBP doses produced no apparent clinical signs. The 30 ug dose of MBP produced the most severe clinical signs, compared to the 1 ug dose. This effect was highly significant ($p < 0.001$, Mann-Whitney U-test). In general increasing the dose (1-30μg) of MBP produced clinical signs earlier, for example 1ug MBP had a mean $\pm$ S.E.M. onset of 14.88 $\pm$ 0.42 (n=9) compared to 12.35 $\pm$ 0.16 (n=34; $p<0.01$) days for the 30ug MBP dose. In addition, a dose dependent effect of MBP (1-30ug) on weight loss was observed. Animals spontaneously recovered from the clinical signs within 5-7 days of onset. Administration of CFA alone produced no clinical signs, however, there was an initial transient weight loss compared to non-treated controls.

In all of these studies no significant differences at any of the MBP doses were observed between the no drug (MBP immunized only) and vehicle dosed groups (MBP immunized and dosed with PBS). Thus, vehicle had no effect on the severity of the disease (see Tables 3 and 4). The no drug and vehicle dosed groups are described below.

Treatment of EAE Various doses of TNF inhibitor dumbbell (0.1 - 3 mg/kg) or vehicle (PBS) at various time courses were administered by subcutaneous injection. Treatment periods began either immediately after or nine days after immunization with MBP and continuing until 21 days post immunization. In each experiment, the control rats receiving PBS received the same number of injections as the treatment groups to diminish any secondary effects due to stress. A group of rats receiving no injections whatsoever after EAE induction, the no drug control, was also observed.

Effects of treatment **Every day dosing** The effects on EAE of everyday dosing with the TNF inhibitor dumbbell, starting on the day of immunization for a total of 21 days, was evaluated. Dumbbell concentrations of 0.1, 0.3, 1 or 3mg/kg had no significant effects on reducing severity of the clinical signs in the 1ug and 30ug MBP groups. However, significant amelioration of the clinical disease was observed at the 3ug MBP dose for all dumbbell doses used.

Every other day dosing The effects of 0.1, 0.3, 1 and 3 mg/kg doses given every other day starting on day nine post immunization were also tested. As shown

in Tables 3 and 4, a significant inhibition of clinical signs occurred at doses of 0.3 ($p<0.008$), 1.0 ($p<0.001$) and 3.0mg/kg ($p<0.002$, Mann Whitney test, $n=6$) compared to vehicle controls using the highest MBP dose (30ug/0.1mL). No significant differences between the vehicle and the no treatment control groups were observed. The lowest dose of the TNF inhibitor dumbbell had no significant effect on clinical signs.

Dumbbell doses of 1.0 ($p<0.1$) and 3mg/kg ($p<0.05$, Mann Whitney test) significantly attenuated the clinical signs produced by 10ug MBP. Although 0.3 and 0.1mg/kg dumbbell attenuated the clinical signs the reduction was not significant. Dumbbell doses of 0.1-3mg/kg did not significantly inhibit the clinical signs induced by lower doses of MBP (1 or 3ug).

Weight loss is an important marker of EAE onset. Rats immunized with 3, 10, and 30 ug MBP that received the c105 dumbbell (1 or 3mg/kg) lost less weight compared to the vehicle groups.

TABLE 3. PREVENTION OF ACTIVELY-INDUCED EAE WITH TNF INHIBITOR DUMBBELL

TABLES 3A - 3F

EFFECTS OF DUMBBELL ON DAILY MEAN CLINICAL SCORE - 30μg MBP

TABLE 3A

Treatment	no drug						
Mean Clinical Score	0.25 ± 0.18	1.00 ± 0.50	1.92 ± 0.52	2.67 ± 0.44	1.83 ± 0.53	0.83 ± 0.44	0.166 ± 0.10
Days	11	12	13	14	15	16	17

TABLE 3B

Treatment	vehicle						
Mean Clinical Score	0.17 ± 0.17	0.75 ± 0.31	1.83 ± 0.40	2.50 ± 0.34	2.08 ± 0.45	1.00 ± 0.41	0.25 ± 0.11
Days	11	12	13	14	15	16	17

TABLE 3C

Treatment	0.1 mg/kg dumbbell						
Mean Clinical Score	0.08 ± 0.08	0.92 ± 0.35	1.33 ± 0.21	2.67 ± 0.21	2.17 ± 0.30	1.17 ± 0.28	0.25 ± 0.11
Days	11	12	13	14	15	16	17

TABLE 3D

Treatment	0.3 mg/kg dumbbell					
Mean Clinical Score	0.25 ± 0.17	0.92 ± 0.27	1.50 ± 0.42	1.17 ± 0.40	0.58 ± 0.15	0.375 ± 0.14
Days	12	13	14	15	16	17

TABLE 3E

Treatment	1 mg/kg dumbbell					
Mean Clinical Score	0.17 ± 0.17	0.58 ± 0.20	0.67 ± 0.17	0.42 ± 0.20	0.33 ± 0.17	0.083 ± 0.083
Days	12	13	14	15	16	17

TABLE 3F

Treatment	3 mg/kg dumbbell					
Mean Clinical Score	0.25 ± 0.17	0.42 ± 0.20	0.83 ± 0.25	0.42 ± 0.32	0.08 ± 0.08	0.08 ± 0.08
Days	12	13	14	15	16	17

table legend: Daily mean severity score in rats immunized with 30ug MBP and treated with TNF inhibitor dumbbell every other day starting 9 days post MBP-immunization. Vehicle group received PBS and the no drug group received no injections post EAE induction.

TABLE 4. INHIBITORY EFFECTS OF TNF INHIBITOR DUMBBELL EXPRESSED AS AREA UNDER CURVE

Treatment	no drug	vehicle	0.1mg/kg	0.3mg/kg	1mg/kg	3mg/kg
Clinical Severity (Area)	8.07 ± 1.40	7.83 ± 0.88	7.88 ± 0.83	4.3 ± 1.02	1.63 ± 0.60	1.53 ± 1.01

table legend: Inhibitory effects of c105 dumbbell on clinical severity expressed as area under curve (units arbitrary). Mean ±S.E.M. (n=6) were determined for each group and compared statistically against the vehicle group (Mann-Whitney test). No significant differences between the vehicle and no drug control group were observed. c105 dumbbell at 0.3, 1.0 and 3.0 mg/kg (given as described above) significantly (**p <0.008, 0.001, and 0.002 respectively) reduced clinical signs.

As shown in Table 5, every other day dosing also reduced the duration of the disease as measured by the number of days during which any clinical signs were observed and the mean calculated for a given group of rats.

TABLE 5. DURATION OF THE DISEASE WITH EVERY OTHER DAY DOSING

MBPμg	TNF inhibitor dumbbell mg/kg				
	0	0.1	0.3	1	3
30	5.33 ± 0.21	5.50 ± 0.34	4.50 ± 0.92	2.83 ± 0.79*	2.16 ± 0.60**
10	4.33 ± 0.80	3.66 ± 0.80	4.00 ± 0.51	3.33 ± 0.49	1.83 ± 0.70*
3	2.50 ± 1.02	1.83 ± 0.83	2.00 ± 0.81	3.16 ± 0.74	0.83 ± 0.54
1	1.83 ± 0.79	0.66 ± 0.66	1.66 ± 0.61	1.33 ± 0.49	0.66 ± 0.42

* $p < 0.05$ ** $p < 0.01$

Single dosing A single dose of either 0.3 or 3mg/kg dumbbell administered on day nine post immunization had little or no effect on attenuating MBP (1-30μg) induced clinical signs when compared to vehicle controls.

Every third day administration of TNF inhibitor dumbbell Dumbbell at 0.1-3mg/kg or vehicle was administered on days 9, 12, 15 and 18 post MBP-immunization. As shown in Table 6, a significant attenuation of MBP (30μg) induced clinical signs was observed at c105 dumbbell doses of 0.3 ($p<0.05$), 1.0 ($p<0.01$) and 3mg/kg ($p<0.001$ Mann-Whitney t-test). The 0.1mg/kg dose of c105 dumbbell was without effect when compared to the vehicle control.

The MBP (10μg) induced clinical signs were reduced by 0.3, 1.0 and 3.0mg/kg c105 dumbbell doses. However, significant ($p<0.05$ and 0.03 respectively) effects were only observed at the higher c105 dumbbell doses. Although c105 dumbbell (0.3-3mg/kg) reduced the clinical signs produced by 3ug of MBP by approximately 20-60%, the effects observed were not significantly different from the vehicle control group.

The duration of the disease was generally reduced by c105 dumbbell. For example, c105 dumbbell at 1 and 3mg/kg significantly reduced the duration MBP

(30μg) mediated signs by 37.3% and 68.7% respectively (see Table 10). A similar trend was also observed using the intermediate MBP (10μg) dose but not the lowest MBP dose (Table 7).

Disease onset in the 10 and 30 μg MBP groups were significantly ($p<0.047$; $p<0.013$ respectively; Mann Whitney U-test) delayed in those animals that were treated with 3mg/kg c105 dumbbell.

The weight loss associated with EAE was partially inhibited by c105 dumbbell especially at the 1 and 3mg/kg doses. The reduction in weight loss was dose dependent. This effect of c105 dumbbell was similar no matter what dose of MBP was used.

TABLE 6. MEAN CLINICAL SEVERITY EXPRESSED AS AREA FOR EVERY THIRD DAY DOSING

Treatment	Vehicle	0.1mg/kg	03.mg/kg	1.0mg/kg	3.0mg/kg
Mean Clinical Severity (Area)	9.21 ±0.64	8.25 ±0.92	6.23 ±1.37	3.66 ±0.61	0.33 ±0.17

TABLE 7. DURATION OF THE DISEASE WITH EVERY THIRD DAY DOSING

MBPμg	TNF inhibitor dumbbell mg/kg				
	0	0.1	0.3	1	3
30	5.83 ± 0.44	4.83 ± 0.30	4.16 ± 0.70	3.66 ± 0.61*	1.83 ± 0.70**
10	4.66 ± 0.42	5.16 ± 0.40	4.00 ± 0.77	3.00 ± 0.96	2.50 ± 0.67*
3	4.00 ± 0.67	3.50 ± 0.62	3.00 ± 1.35	3.00 ± 1.35	3.33 ± 0.66

* $p < 0.05$ ** $p < 0.01$

EXAMPLE 18. Central Nervous System (CNS) Pathology

The effects of treatment with c105 dumbbell synthesized using PEG-*bis*-vinyl sulfone were determined on CNS pathology induced by immunization with MBP (0, 10 or 30μg). MBP-immunization (EAE induction) was performed as described above. c105 dumbbell at 0.3, 3mg/kg or vehicle was administered every other day beginning on day nine post MBP. Animals were killed (via CO_2) on days 9, 14 or 20 post-MBP injection. The brain and spinal cord from each rat were removed and placed in 10% neutral buffered formalin. Following fixation for at least 72 hours, cross sections of the brain were made at the level of the optic chiasm caudal to the attachment of the pituitary and the transverse fibers of the pons. The spinal cord was trimmed by making 4-6 cross sections through the cervical, thoracic and lumber portions. The sacral segment with attached caudal nerves was embedded longitudinally. Tissues were processed for paraffin embedding and stained with hematoxylin and eosin.

Histologic evaluations were done without knowledge of the treatment groups. Each slide was assigned a numerical score ranging from 1-4 to indicate the intensity of inflammation and demyelination. Scoring criteria were as follows; 1=minimal 1-2 vessels have small perivascular cuffs of inflammatory cells, 2=mild 3 or more vessels have small perivascular cuffs of inflammatory cells with little if any extension of inflammation into parenchyma, 3=moderate 3 or more vessels have prominent perivascular cuffs of inflammatory cells with moderate extension of the inflammation into the surrounding parenchyma, and 4=marked the majority of vessels have prominent perivascular cuffs of inflammatory cells with extensive involvement of the neuropil in the inflammatory process.

Total inflammation scores were determined for each of animals for each CNS region. Mean $\pm$ SEM (standard error of the mean) score values were computed for each portion of the CNS for each time point and compared against the vehicle treated animals.

The mean inflammatory score were determined for each CNS region for each group of animals and compared statistically against the vehicle control group (students-t-test). These scores are set forth in Tables 8 and 9.

There were no significant histologic alterations in the CNS of animals killed at day 9 post-MBP injection. Lesions at day 14 consisted of minimal to marked mixed (mononuclear + some neutrophils) generally perivascular inflammatory cell infiltration. In the brain, the inflammation tended to be located in the meninges, periventricular areas and cerebellar white tracts, with the brain stem and cerebellar white tracts being most severely affected. In these locations, the inflammation often extended from perivascular areas into the surrounding parenchyma and there was evidence of demyelination. Within the spinal cord, the lumbar and sacral portions were most severely affected. Both gray and white matter were affected, again with the predominant lesion being perivascular. Inflammation persisted into day 20, however, neutrophils were rarely seen at this time point. Variability in intensity of inflammation occurred within animals in each group and almost all group.

Tables 8 and 9 demonstrate the presence of c105 dumbbell reduced the degree of inflammation in the various regions of the CNS studied. The most dramatic and significant reductions in inflammation were observed in the spinal cord, particularly the lumbar and sacral regions. c105 dumbbell had a lesser effect on the higher regions of the CNS, cerebrum and cerebellum.

TABLE 8. INFLAMMATORY SCORES OF ANIMALS IMMUNIZED WITH 30UG MBP AND TREATED WITH TNF INHIBITOR DUMBBELL

Brain Region	3mg/kg	0.3mg/kg	Vehicle
Cerebrum	1.00 ± 0.378	0.714 ± 0.360	0.714 ± 0.474
Cerebellum	2.57 ± 0.429	2.714 ± 0.360	3.280 ± 0.286
Cervical cord	1.71 ± 0.360	1.428 ± 0.298*	2.420 ± 0.202
Thoracic cord	1.71 ± 0.421	1.000 ± 0.218*	2.280 ± 0.421
Lumbar cord	1.85 ± 0.404	1.42 ± 0.369	2.42 ± 0.298
Sacral cord	1.28 ± 0.360	1.42 ± 0.298	2.714 ± 0.522

* $p < 0.05$ (Students t-test) Histology (30ug MBP dose)

TABLE 9. INFLAMMATORY SCORES OF ANIMALS IMMUNIZED WITH 10UG MBP AND TREATED WITH TNF INHIBITOR DUMBBELL

Brain Region	3mg/kg	0.3mg/kg	Vehicle
Cerebrum	0.28 ± 0.18	0.42 ± 0.20	0.42 ± 0.29
Cerebellum	1.42 ± 0.29	2.28 ± 0.42	2.28 ± 0.35
Cervical cord	0.85 ± 0.34	1.42 ± 0.42	1.42 ± 0.20
Thoracic cord	0.85 ± 0.14	1.57 ± 0.36	1.0 ± 0.30
Lumbar cord	0.71 ± 0.28**	1.57 ± 0.48	2.28 ± 0.28
Sacral cord	0.57 ± 0.20**	1.57 ± 0.48	2.28 ± 0.42

** $p < 0.01$ Histology (10ug MBP dose)

EXAMPLE 19. c105 TNFbp dumbbell protects against endotoxin lethality

The c105 dumbbell synthesized using PEG-*bis*-vinyl sulfone protected Balb/c mice against a lethal dose of endotoxin. Mice were injected intraperitoneally with 30 mg/kg endotoxin and intravenously with a single administration of either 0.1 mL PBS or 1 mg/kg dumbbell in 0.1 mL PBS at either 1 hour or two hours after the administration of endotoxin. The intravenous administration of 1 mg/kg dumbbell 1 hour after injection of endotoxin caused almost complete protection against lethality. Dumbbell administration at the two hour time point gave no protection against the lethal endotoxin injury.

The c105 dumbbell also protected Lewis rats against a lethal dose of endotoxin. Rats were injected intravenously with 12.5 mg/kg endotoxin. Rats were injected simultaneously with endotoxin and either saline or the c105 dumbbell at doses of either 0.1, 0.5, 3.0 or 4.5 mg/kg. Comparable protection against lethal injury was achieved at all dumbbell doses.

A single dose treatment of 1.5 mg/kg c105 dumbbell given simultaneously with a 10 mg/kg dose of endotoxin protected rats against hepatic and metabolic disturbances. Hepatic and metabolic parameters were assessed at 24 hours after the administration of endotoxin as shown in Table 10.

TABLE 10: EFFECTS OF TREATMENT WITH c105 DUMBBELL (1.5 MG/KG) ON ENDOTOXIN-INDUCED ABNORMALITIES IN BIOCHEMICAL PARAMETERS

Parameter	Control + Vehicle	Endotoxin + Vehicle	Endotoxin + c105 dumbbell
Glucose (mg/dL)	143 ± 2	52 ± 8	81 ± 5*
SGPT[1] (mu/mL)	47 ± 6	679 ± 118	141 ± 25*
Blood Urea Nitrogen (mg/dL)	19 ± 1	88 ± 2	39 ± 3*
Corticosterone (ng/mL)	164 ± 62	750 ± 49	489 ± 43*

[1] Serum Glutamic Pyruvic Transaminase
Values are means ± standard error for 4 to 8 rats per group.
*Significantly different from the endotoxin-treated group at $p < 0.05$ (paired t test)

It is to be understood that the application of the teachings of the present invention to a specific expression system or PEGylation reagent will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Thus, it will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the process and products of the present invention. It is intended that the present invention covers these modifications and variations.

What is claimed is:

1. A biologically-active conjugate comprising:

a biologically-active molecule selected from the group consisting of an IL-1 inhibitor, a tumor necrosis factor (TNF) inhibitor, CR1, PDGF receptor, IL-2, and exon 6 peptide of PDGF, wherein said biologically-active molecule has a reactive thiol moiety, and

a non-peptidic polymer having an active sulfone moiety forming a linkage with said thiol moiety.

2. The biologically-active conjugate of claim 1, wherein said active sulfone moiety is vinyl sulfone.

3. The biologically-active conjugate of claim 1, wherein said active sulfone moiety is chloroethyl sulfone.

4. The biologically-active conjugate of claim 1, wherein said biologically-active molecule is a TNF inhibitor selected from the group consisting of a 30kDa TNF inhibitor, a 40kDa TNF inhibitor, a Δ51 TNF inhibitor, and a Δ53 TNF inhibitor.

5. The biologically-active conjugate of claim 4, wherein said TNF inhibitor is the 30kDa TNF inhibitor.

6. The biologically-active conjugate of claim 4, wherein said TNF inhibitor is the 40kDa TNF inhibitor.

7. The biologically-active conjugate of claim 4, wherein said TNF inhibitor is the Δ51 TNF inhibitor.

8. The biologically-active conjugate of claim 4, where in said TNF inhibitor is the Δ53 TNF inhibitor.

9. The biologically-active conjugate of claim 1, wherein said biologically-active molecule is an interleukin-1 (IL-1) inhibitor.

10. The biologically-active conjugate of claim 9, wherein said IL-1 inhibitor is interleukin-1 receptor antagonist (IL-1ra).

11. The biologically-active conjugate of claim 1, wherein said non-peptidic polymer has a reactive NHS-ester in addition to said active sulfone moiety.

12. The biologically-active conjugate of claim 11, wherein said active sulfone moiety is vinyl sulfone.

13. A substantially purified compound of the formula R_1-X-R_2, wherein:

X comprises a non-peptidic polymer having a first reactive group and a second reactive group, wherein said first reactive group is a Michael acceptor;

R_1 comprises a biologically-active molecule selected from the group consisting of an IL-1 inhibitor, a tumor necrosis factor (TNF) inhibitor, CR1, PDGF receptor, IL-2, and exon 6 peptide of PDGF, has a reactive thiol moiety, said biologically-active molecule is covalently bonded to said non-peptidic polymer by reaction of said thiol moiety with said Michael acceptor, and said biologically-active molecule retains its biological activity after said reaction; and

R_2 comprises a biologically-active molecule or a nonbiologically-active group bonded to said non-peptidic polymer by reaction with said second reactive group.

14. The substantially purified compound of claim 13, wherein said Michael acceptor is vinyl sulfone.

15. The substantially purified compound of claim 13, wherein said second reactive group is an NHS-ester.

16. The substantially purified compound of claims 13 or 15, wherein said Michael acceptor is maleimide.

17. The substantially purified compound of claim 13, wherein said non-peptidic polymer has two Michael acceptors.

18. The substantially purified compound of claim 17, wherein said Michael acceptors are maleimide.

19. The substantially purified compound of claim 17, wherein said Michael acceptors are vinyl sulfone.

20. The substantially purified compound of claim 17, wherein one of said Michael acceptors is vinyl sulfone and the other is maleimide.

21. The substantially purified compound of claim 13, wherein said biologically-active molecule is a TNF inhibitor.

22. The substantially purified compound of claim 21, wherein said TNFbp is the 30kDa TNF inhibitor.

23. The substantially purified compound of claim 21, wherein said TNFbp is the 40kDa TNF inhibitor.

24. The substantially purified compound of claim 21, wherein said TNFbp is the Δ51 TNF inhibitor.

25. The substantially purified compound of claim 21, wherein said TNFbp is the Δ53 TNF inhibitor.

26. The substantially purified compound of claim 13, wherein said biologically-active molecule is an IL-1 inhibitor.

27. The substantially purified compound of claim 26, wherein said IL-1 inhibitor is IL-1ra.

28. A water soluble polymer having a reactive NHS-ester and a maleimide.

29. The water soluble polymer of claim 28, wherein said polymer is selected from the group consisting of polyalkylene oxides, polyoxyethylated polyols, and polyolefinic alcohols.

30. A pharmaceutical composition comprising the compound of claim 1 in a pharmaceutically-acceptable carrier.

31. A pharmaceutical composition comprising the compound of claim 13 in a pharmaceutically-acceptable carrier.

INTERNATIONAL SEARCH REPORT

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PCT/US 95/07555

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO-A-92 16221 (SYNERGEN INC) 1 October 1992 cited in the application see page 12, line 33 - page 14, line 18 see page 18, line 19 - line 35 see page 25, line 17 - line 35 see page 28, line 6 - page 29, line 28 see page 30, line 12 - line 27; claims --- -/--	1,4-10, 13, 15-18, 21-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO-A-93 01498 (IMMUNODEX K S) 21 January 1993 see page 1, line 11 - line 16 see page 20, line 22 - page 21, line 26 see page 18, line 3 - line 33; claims 1,2,5,7,18	1,2, 9-21, 26-31
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Intern al Application No
PCT/US 95/07555

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Chemistry for peptide and protein PEGylation

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Abstract

Poly(ethylene glycol) (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. In the modification of biological macromolecules, peptides and proteins are of extreme importance. Reasons for PEGylation (i.e. the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing the renal filtration and altering biodistribution. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein. In this paper, we review PEG chemistry and methods of preparation with a particular focus on new (second-generation) PEG derivatives, reversible conjugation and PEG structures.

Keywords: PEGylation; PEG-protein; PEG conjugation; PEG chemistry

Contents

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1. Introduction

The use of proteins and peptides as human therapeutics has expanded in recent years due to: (1) discovery of novel peptides and proteins, (2) a better understanding of the mechanism of action in vivo, (3) improvements in expression or synthesis of proteins and peptides that closely resemble fully human proteins and peptides, and (4) improvements in formulation or molecule-altering technologies that have the ability to deliver polypeptides in vivo with improved pharmacokinetic and pharmacodynamic properties. It was estimated that in the year 2000, as many as 500 biopharmaceutical products were undergoing clinical trials, and the estimated annual growth rates of protein products (glycoproteins, unglycosylated proteins and antibodies) will range from 10 to 35% [1].

Although more biopharmaceuticals are in development than ever before, many of these have problems that are typical of polypeptide therapeutics, including short circulating half-life, immunogenicity, proteolytic degradation, and low solubility. Several strategies have emerged as ways to improve the pharmacokinetic and pharmacodynamic properties of biopharmaceuticals, including: (1) manipulation of amino acid sequence to decrease immunogenicity and proteolytic cleavage, (2) fusion or conjugation to immunoglobulins and serum proteins, such as albumin, (3) incorporation into drug delivery vehicles for protection and slow release, and (4) conjugating to natural or synthetic polymers [2–6].

Those in the biomedical, biotechnical and pharmaceutical communities have become quite familiar with the improved pharmacological and biological properties that are associated with the covalent attachment of poly(ethylene glycol) or PEG to therapeutically useful polypeptides. For instance, PEG conjugation can shield antigenic epitopes of the polypeptide, thus reducing reticuloendothelial (RES) clearance and recognition by the immune system and also reducing degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing renal filtration and altering biodistribution. Contributing factors that affect the foregoing properties are: (1) the number of PEG chains attached to the polypeptide, (2) the molecular weight and structure of PEG chains attached to the polypeptide, (3) the location of the PEG sites on the polypeptide and (4) the chemistry used to attach the PEG to the polypeptide.

The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates have reached late phase clinical trials. The first few PEG-protein products, now on the market (Adagen®, Oncospar®, and PEG-Intron®), were developed using first generation PEG chemistry. One characteristic of first generation PEG chemistry is the use of low molecular weight linear PEGs ($\leq$ 12 kDa) with chemistry that may result in side reactions or weak linkages upon conjugation with polypeptides.

The next generation of PEG-protein therapeutics, which will come to market in the next several years, uses second-generation PEG chemistries. Second-generation PEGylation was designed to avoid the problems of first generation chemistry, notably diactivated PEG impurities, restriction to low molecular weight mPEG, unstable linkages and lack of selectivity in modification. Readers are referred to several detailed reviews on different aspects of PEGylation [7–11]. In this paper, we review chemistries of both first- and second-generation, with an emphasis on newer PEGylation technologies, in order to provide an introduction to those chemistries that will be used in the following reviews.

2. Properties of PEG

In its most common form poly(ethylene glycol), PEG, is a linear or branched polyether terminated with hydroxyl groups and having the general structure:

$$HO–(CH_2CH_2O)_n–CH_2CH_2–OH$$

PEG is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Most useful for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:

$$CH_3O–(CH_2CH_2O)_n–CH_2CH_2–OH$$

Monomethoxy PEG is synthesized by anionic ring opening polymerization initiated with methoxide ions. Commercially available mPEG contains a considerable amount of diol PEG due to the presence of trace amounts of water during polymerization. This diol PEG is also of relatively high molecular weight due to polymerization at both ends of the polymer. The amount of diol PEG can exceed 15% of the composition of mPEG. A solution to the problem of diol contamination has been developed in our laboratories [12]. In this work, a crude benzyloxy-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus diol is converted to the inert dimethyl ether, which can be subsequently removed after activation and polypeptide attachment.

$$BzO\text{–}PEG\text{–}OH + HO\text{–}PEG\text{–}OH \rightarrow \rightarrow HO\text{–}PEG\text{–}OCH_3 + CH_3O\text{–}PEG\text{–}OCH_3$$

Another common route to remove diol is to convert the PEGs to PEG-carboxylic acids that can then be purified by ion-exchange chromatography [13]. PEG with various end groups can be prepared by use of suitable initiator and/or termination reagents. Numerous functionalities can be introduced as end groups on PEG in this manner, including heterobifunctional products. For instance, Kataoka et al. synthesized a heterobifunctional PEG derivative containing aldehyde and thiol end groups [14]. Polymerization was initiated with 3,3-diethoxy-1-propanol, which forms a propionaldehyde after acid hydrolysis, and the polymerization was terminated with methansulfonyl chloride with successive conversion to ethyldithiocarbonate and a free thiol.

Compared with other polymers, PEG has a relatively narrow polydispersity (M_w/M_n) in the range of 1.01 for low molecular weight PEGs (<5 kDa) to 1.1 for high molecular weight PEGs (>50 kDa).

The unique ability of PEG to be soluble in both aqueous solutions and organic solvents makes it suitable for end group derivatization and chemical conjugation to biological molecules under mild physiological conditions. Studies of PEG in solution have shown that PEG typically binds 2–3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule acts as if it were five to 10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins [15], exclude proteins and cells from surfaces [16], reduce immunogenicity and antigenicity [17] and prevent degradation by mammalian cells and enzymes [18].

Low molecular weight oligomers of PEG (<400 Da) have been shown to be degraded in vivo by alcohol dehydrogenase to toxic metabolites, however the lack of toxicity of PEGs with a molecular weight above 1000 Da has been revealed over many years of use in foods, cosmetics and pharmaceuticals [18].

PEG is rapidly cleared in vivo without structural change and clearance is dependent on molecular weight. Below a molecular weight of about 20 kDa the molecule is cleared in the urine, and higher molecular weight PEGs are cleared more slowly in the urine and feces. PEG is only weakly immunogenic even at high molecular weights. Antibodies to PEG have been generated when attached to a highly immunogenic molecule under an immunization protocol with Freund's adjuvant [19–21]. There are no known situations in which anti-PEG antibodies have been generated under 'normal' clinical administration of a PEG-modified protein.

3. Chemistry of pegylation

To couple PEG to a molecule (i.e. polypeptides, polysaccharides, polynucleotides and small organic molecules) it is necessary to activate the PEG by preparing a derivative of the PEG having a functional group at one or both termini. The functional group is chosen based on the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid. In the case of glycoproteins, vicinal hydroxyl groups can be oxidized with periodate to form two reactive formyl moieties.

The most common route for PEG conjugation of proteins has been to activate the PEG with functional groups suitable for reaction with lysine and N-terminal amino acid groups. Lysine is one of the most

prevalent amino acids in proteins and can be upwards of 10% of the overall amino acid sequence. In reactions between electrophilically activated PEG and nucleophilic amino acids, it is typical that several amines are substituted. When multiple lysines have been modified, a heterogeneous mixture is produced that is composed of a population of several polyethylene glycol molecules attached per protein molecule ('PEGmers') ranging from zero to the number of ε- and α-amine groups in the protein. For a protein molecule that has a single PEG attached by this nonspecific modification method, the polyethylene glycol moiety may be attached at a number of different amine sites. Therefore there is the potential for a large number of positional isomers (P) as the degree of modification increases:

$$P = \frac{N!}{(N-k)! \times k!}$$

where N is the number of possible sites and k is the number of sites modified. The extent of modification is important in determining the pharmacological properties of the bioconjugate. Typically, a higher degree of modification will extend the circulation half-life and reduce the likelihood of antigenicity [22]. Each positional isomer of the heterogeneous mixture is likely to have an influence on whether the conjugate is active or whether an antibody will bind an antigenic epitope. The heterogeneity in lysine substitution and in PEG molecular weights is of some concern for PEG-protein pharmaceuticals, and it is generally necessary to demonstrate that the pattern for a particular pharmaceutical can be measured and is reproducible. Many of the important benefits of PEGylation can be controlled by proper conjugation of various molecular weight PEGs to the protein at specific locations on the protein's surface.

The monofunctionality of methoxyPEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce crosslinked polypeptides, as long as diol PEG has been removed. As we will see in the discussion of second generation PEGylation, it is also possible in some instances to reduce or eliminate heterogeneity in the position of substitution.

3.1. First-generation PEG chemistry

3.1.1. PEG chemistry for amine conjugation

Since most applications of PEG conjugation involve labile molecules, the coupling reactions require mild chemical conditions. In the case of polypeptides, the most common reactive groups involved in coupling are the alpha or epsilon amino groups of lysine. In Fig. 1 is listed a wide range of first generation PEG derivatives used for protein PEGylation of either the alpha or epsilon amino groups. First-generation chemistries are generally plagued by PEG impurities, restriction to low molecular weights, unstable linkages, and lack of selectivity in modification. Examples of first-generation PEG derivatives include: (a) PEG dichlorotriazine, (b) PEG tresylate, (c) PEG succinimidyl carbonate, (d) PEG benzotriazole carbonate, (e) PEG p-nitrophenyl carbonate, (f) PEG trichlorophenyl carbonate, (g) PEG carbonylimidazole and (h) PEG succinimidyl succinate.

The initial work of Davis et al. used cyanuric chloride to prepare activated PEG for attachment to proteins [6,17]. The PEG dichlorotriazine (Fig. 1a) derivative can react with multiple nucleophilic functional groups such as lysine, serine, tyrosine, cysteine, and histidine, which results in displacement of one of the chlorides and produces a conjugate with retained charge in the form of a secondary amine linkage [23]. The remaining chloride is less susceptible to reactions with nucleophilic residues. Unfortunately, the reactivity is sufficient to allow crosslinking of protein molecules containing additional nucleophilic residues. To solve this problem, Inada et al. synthesized 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine ($mPEG_2$-chlorotriazine) as shown in Fig. 2 [24]. The lower reactivity of the remaining chlorine translates into a more selective modification of lysine and cysteine residues without further side reactions.

Another alkylating reagent used to nonspecifically modify multiple amino groups to form secondary amine linkages to proteins, viruses and liposomes is PEG tresylate (Fig. 1b) [25]. Although more specific to amino groups than PEG dichlorotriazine, the chemistry of conjugation and the conjugation products are not unique and well defined. For example,

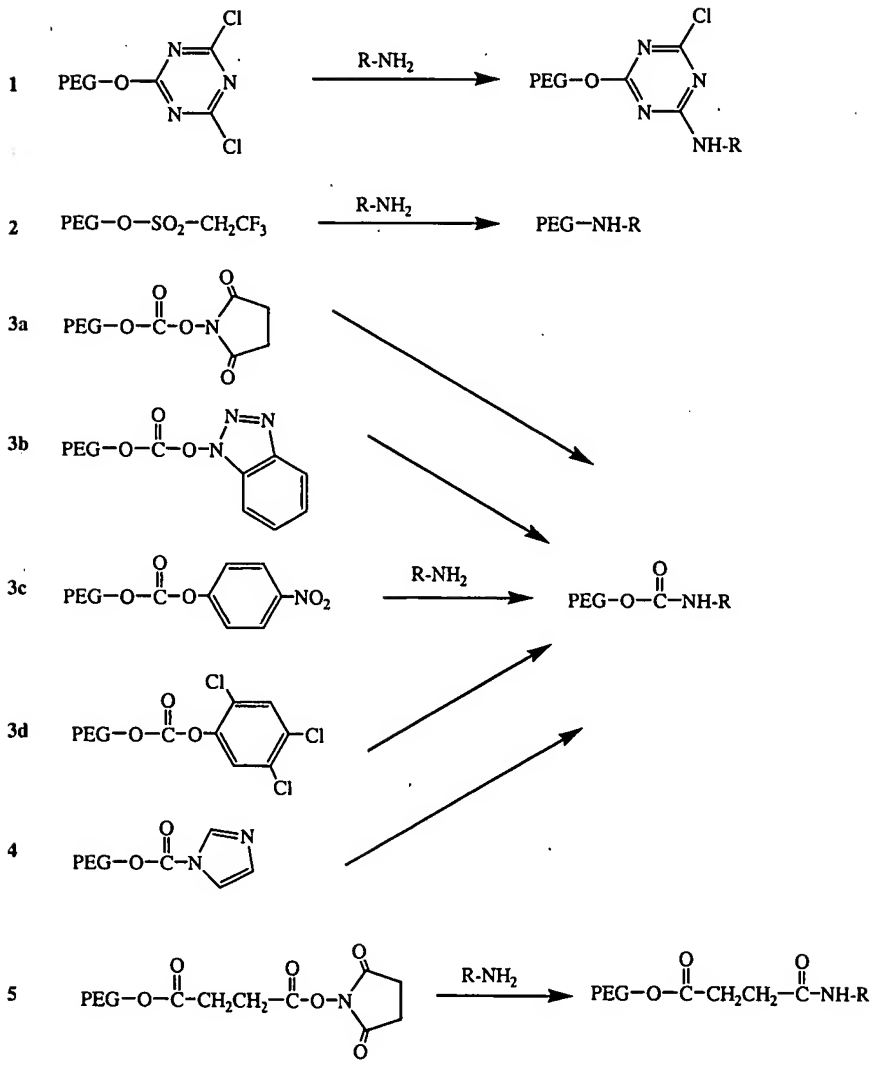

Fig. 1. First-generation amine reactive PEG derivatives.

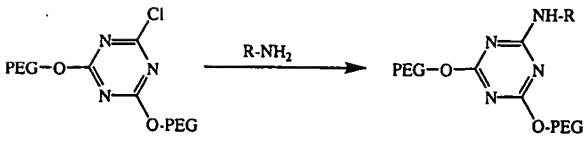

Fig. 2. Branched PEG (PEG2) based on PEG-triazine.

Gais et al. have shown that PEG-tresylate conjugation to small molecule amines can produce a product that contains a degradable sulfamate linkage [26]. Therefore, a heterogeneous mixture that results from attaching PEG-tresylate to proteins may contain a population of conjugates with degradable linkages.

Most first-generation PEG chemistries are those that produce conjugates through acylation. Two widely used first-generation activated mPEGs are succinimidyl carbonate (SC-PEG in Fig. 1c) [27,28] and benzotriazole carbonate (BTC-PEG in Fig. 1d) [29]. SC-PEG and BTC-PEG react preferentially with lysine residues to form a carbamate linkage, but are also known to react with histidine and tyrosine residues. SC-PEG is slightly more stable to hy-

drolysis than BTC-PEG with a half-life of 20.4 min at pH 8 and 25 °C compared to the 13.5 min hydrolysis half-life of BTC-PEG under the same conditions [30]. It has recently been observed that SC-PEG and BTC-PEG couple to histidine residues of α-interferon at slightly acidic conditions to form a hydrolytically unstable imidazolecarbamate linkage [31]. The weak linkage could be used to advantage in preparation of controlled-release formulation, or it could be a disadvantage if conjugate instability were not desired.

Other PEG acylating reagents which produce urethane linked proteins include *p*-nitrophenyl carbonate (pNPC-PEG in Fig. 1e), trichorophenyl carbonate (TCP-PEG in Fig. 1f) and carbonylimidazole (CDI-PEG in Fig. 1g) [32,33]. These reagents are prepared by reacting chloroformates or carbonylimidazole with the terminal hydroxyl group on mPEG, and these have much lower reactivity than either the SC-PEG or BTC-PEG. Generally, the slower the reaction the more specific the reagent is to certain amino acid groups of the protein. In this way, some selectivity is achieved. The extent and rate of modification can easily be followed in the case of pNPC-PEG and TCP-PEG by monitoring the phenolate-ion leaving-group by colorimetric analysis.

The remaining first-generation PEG reagent is succinimidyl succinate (SS-PEG in Fig. 1h) [34]. SS-PEG is prepared by reaction of mPEG with succinic anhydride, followed by activation of the carboxylic acid to the succinimidyl ester. The polymer backbone contains a second ester linkage that remains after the conjugation reaction with a protein. This linkage is highly susceptible to hydrolysis after the polymer has been attached to the protein. Not only does this hydrolysis lead to loss of the benefits of PEG attachment, but the succinate tag that remains on the protein after hydrolysis can act as a hapten and lead to immunogenicity of the remaining protein [35].

Techniques used to form first generation PEG derivatives are generally straightforward and involve reacting the PEG polymer with a group that is reactive with hydroxyl groups, typically anhydrides, chlorides, chloroformates and carbonates. With the exception of the work by Bentley et al., these techniques lack the ability to produce pure monofunctional PEG derivatives of high molecular weight [12]. Since the diol content of high molecular weight PEGs can reach 15%, high-molecular-weight, first-generation PEG chemistry is inefficient for protein conjugation. The ability to generate an intermediate that can be purified from diactivated species renders second-generation chemistry a valuable tool for protein modification.

3.2. Second-generation PEGylation chemistry

3.2.1. PEG chemistry for amine conjugation

Second-generation PEGylation chemistry has been designed to avoid the above noted problems of diol contamination, restriction to low molecular weight mPEG, unstable linkages, side reactions and lack of selectivity in substitution. One of the first examples of second-generation chemistry is mPEG-propionaldehyde [36]. mPEG-propionaldehyde is easier to prepare and use than PEG-acetaldehyde because the acetaldehyde is very susceptible to dimerization via aldol condensation. A key property of mPEG-priopionaldehyde, as disclosed by Kinstler et al. in work on PEGylation of G-CSF, sTNF-RI, and consensus IFN, is that under acidic conditions (approximately pH 5), aldehyde is largely selective for the N-terminal α-amine because of the lower pK_a of the α-amine compared to other nucleophiles [37–39]. The conjugation of electrophilic PEGs to amino acid residues on proteins is highly dependent on the nucleophilicity of each amino acid residue. Nucleophilic attack will only take place when the pH of the protein solution is near or above the residue's pK_a. Therefore the reactivity of each residue also depends on neighboring amino acid residues. Although complete selectivity is not observed, the extensive heterogeneity frequently seen with lysine chemistry is greatly reduced. Coupling of aldehydes to primary amines proceeds through a Schiff base, which is reduced in situ to give a stable secondary amine linkage as shown in Fig. 3.

An alternative approach to using PEG-aldehyde is to use the acetal derivative of PEG-propionaldehyde or PEG-acetaldehyde [40]. The aldehyde hydrate of the acetal derivatives can be generated in situ by acid hydrolysis (Fig. 4). The pH of the solution can then be adjusted to values sufficient for protein modification with the same mechanism as the free aldehyde derivative in Fig. 3. The benefit of using the

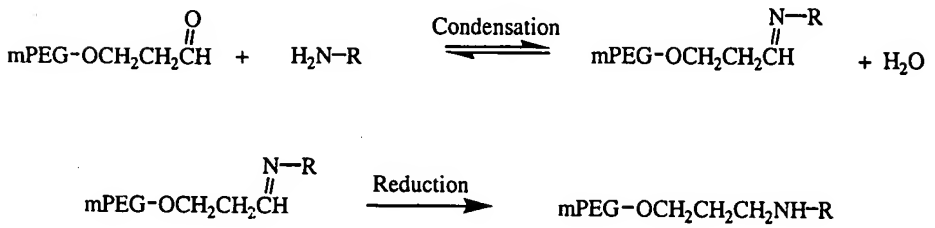

Fig. 3. Reductive amination using PEG-propionaldehyde.

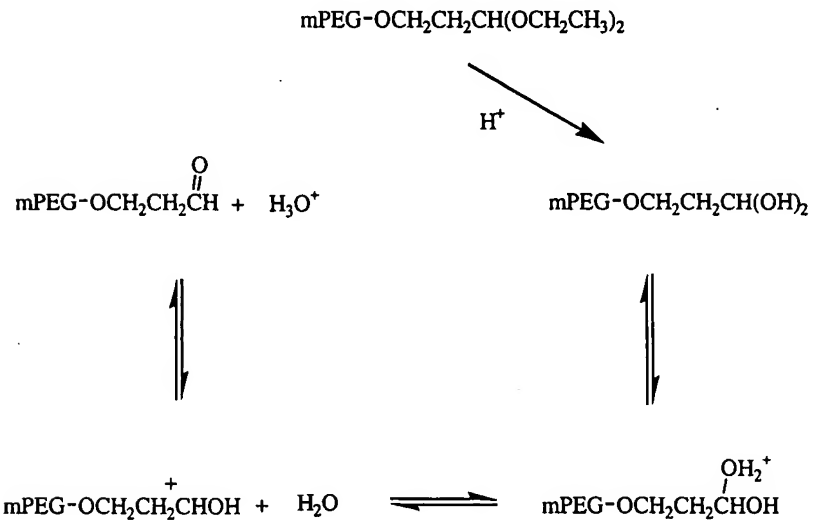

Fig. 4. In situ generation of PEG-aldehyde hydrate for use in reductive amination.

acetal derivative over the free propionaldehyde or acetaldehyde is longer storage stability and higher purity.

Active esters of PEG carboxylic acids are the most used acylating agents for protein modification. Active esters react with primary amines near physiological conditions to form stable amides as shown in Fig. 5. Generating the carboxylic acid intermediate allows the PEG to be purified from unsubstituted or disubstituted impurities by ion-exchange chromatography [41]. Purities of greater than 97% are routinely obtainable by this method. Activation of PEG-carboxylic acids to the succinimidyl active esters is accomplished by reacting the PEG-carboxylic acid with *N*-hydroxysuccinimide (NHS or HOSu) and a carbodiimide.

The first carboxylic acid derivative of PEG not containing a degradable linkage to the PEG backbone, as in SS-PEG, was carboxymethylated PEG (CM-PEG) [42]. The succinimidyl ester of this compound (SCM-PEG) is extremely reactive (hydrolysis $t_{1/2}$ of 0.75 min at pH 8 and 25 °C) and is therefore difficult to use. To take advantage of the intermediate purification step and have an active ester that had more favorable kinetics for protein modification, Harris et al. prepared propionic acid (PEG–O–CH_2CH_2–COOH) and butanoic acid (PEG–O–$CH_2CH_2CH_2$–COOH) derivatives of PEG (Fig. 5(1)) [13]. Changing the distance between the active ester and the PEG backbone by the addition of methylene units had a profound influence on the reactivity towards amines and water. For example, SBA-PEG, which has two additional methylene groups, has a longer hydrolysis half-life of 23 min at pH 8 and 25 °C. SPA-PEG, which has one additional methylene group, has a hydrolysis half-life of 16.5 min at pH 8 and 25 °C.

Reactivity of PEG active esters towards amines

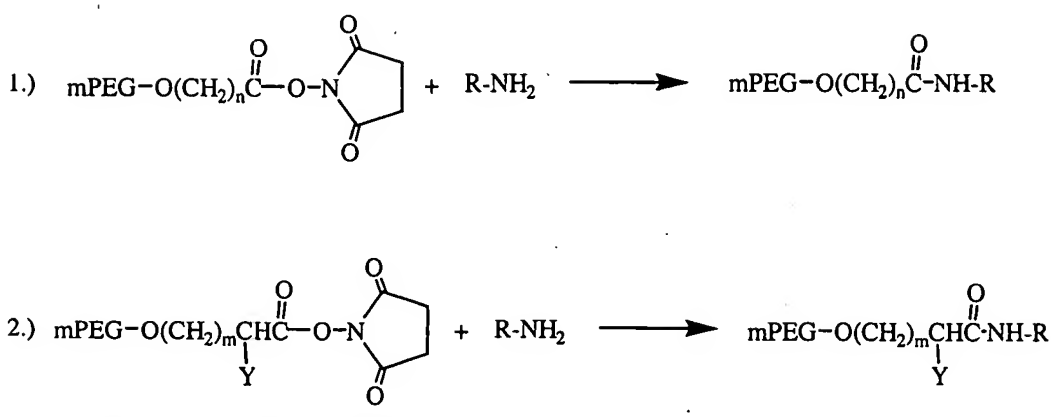

Fig. 5. PEG NHS esters. (1) PEG NHS esters based on propionic and butanoic acids and (2) α-branched PEG NHS esters based on propionic and butanoic acids.

and water can be further decreased by introducing an α-branching moiety to the carboxylic acid as shown in Fig. 5(2). An α-methyl branched PA-PEG derivative has a hydrolysis half-life of 33 min at pH 8 and 25 °C.

3.2.2. PEG chemistry for cysteine modification

PEGylation of free cysteine residues in proteins is the main approach for site-specific modification because reagents that specifically react with cysteines have been synthesized, and the number of free cysteines on the surface of a protein is much less than that of lysine residues. In the absence of a free cysteine in a native protein, one or more free cysteines can be added by genetic engineering [43]. The advantage of this approach is that it makes possible site-specific PEGylation at areas on the protein that will minimize a loss in biological activity but decrease immunogenicity. This strategy is not without its shortcomings. The addition of free cysteines by genetic engineering increases the possibility of incorrect disulfide formation and protein dimerization.

PEG derivatives such as PEG-maleimide (Fig. 6(1)), vinylsulfone (Fig. 6(2)), iodoacetamide (Fig. 6(3)), and orthopyridyl disulfide (Fig. 6(4)) have been developed for PEGylation of cysteine residues, with each derivative having its own advantages and disadvantages [43–46]. PEG-vinylsulfone (PEG-VS) reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH 7–8) but will proceed faster if the pH is increased. Although PEG-VS is stable in aqueous solutions, it may react with lysine residues at elevated pH. Unlike PEG-VS, PEG-maleimide (PEG-MAL) is more reactive to thiols even under acidic conditions (pH 6–7), but it is not stable in water and can undergo ring opening or addition of water across the double bond. PEG-iodoacetamide (PEG-IA) reacts slowly with free thiols by nucleophilic substitution, creating a stable thioether linkage. The reaction should be done in slight molar excess of PEG-IA in a dark container to limit the generation of free iodine that may react with other amino acids. The thioether linkage between the PEG-MAL and protein is stable, but slow cleavage of one of the amide linkages can occur by hydrolysis. Orthopyridyl disulfide-PEG (PEG-OPSS) reacts specifically with sulfhydryl groups under both acidic and basic conditions (pH 3–10) to form a disulfide bond with the protein. Disulfide linkages are also stable, except in a reducing environment when the linkage is converted to thiols.

Scientists in our laboratories recently prepared a highly active, long circulating and stable conjugate of IFN-β using a two-step method with PEG-OPSS [47]. The tertiary structure of IFN-β was determined by Karpusas et al. who showed that the free cysteine residue at position 17 was proximal to the surface but hidden [48]. In this case, the available thiol was not accessible to high molecular weight PEG that would be needed for improved pharmacokinetics. The approach that was ultimately adopted was to couple a low molecular weight di-OPSS PEG (M_w 2000) to the interferon and then couple a PEG thiol

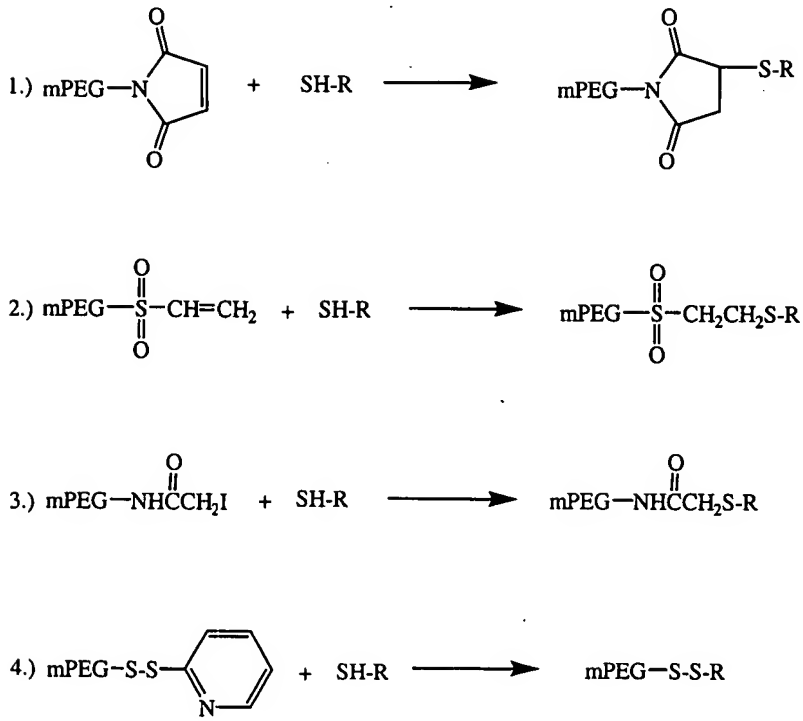

Fig. 6. Thiol reactive PEGs. (1) PEG maleimide, (2) PEG vinyl sulfone, (3) PEG iodoacetamide, and (4) PEG orthopyridyl disulfide.

to the remaining terminal OPSS group. The disulfide linkage between PEG and the protein was found to be stable in plasma circulation (unpublished data).

3.2.3. PEG chemistry for oxidized carbohydrates or N-terminus

Oxidation of carbohydrate residues or N-terminal serine or threonine is an alternative method for site-directed PEGylation of proteins. Carbohydrates can be oxidized with enzymes, such as glucose oxidase, or chemically with sodium periodate. Oxidation of the carbohydrate residues generates multiple reactive aldehyde groups, which can be reacted with either PEG-hydrazide to produce a hydrazone linkage or with PEG-amine to produce a reversible Schiff base (Fig. 7) [49]. The hydrazone linkage may be reduced with sodium cyanoborohydride to a more stable alkyl hydrazide and the Schiff's base may be reduced to form a secondary amine. Reductive alkylation with PEG-amine is problematic because the amino groups of a protein possess similar reactivity to PEG-amines and thus may form cross-linked aggregates. PEG-hydrazides are more useful in these situations. Under acidic conditions (approx. pH 5), amino groups of the protein are predominantly protonated, but because the PEG-hydrazide is a weaker base (pK_a approx. 3) than primary amines (pK_a approx. 10), the reaction is selective to the PEG-hydrazone formation. Multiple attachment sites are generated using this method, but the modification site is specific to the carbohydrate.

Another approach to site-specific conjugation is to take advantage of the presence of a N-terminal serine or threonine, which can be converted by periodate oxidation to a glyoxylyl derivative. Gaertner et al. oxidized the N-terminal serine of IL-8 to form a glyoxylyl derivative, which they conjugated to aminooxy and hydrazide PEG derivatives [50].

3.2.4. PEG chemistry for reversible PEGylation

Most PEGylation chemistry is designed to create a conjugate that contains a stable linkage to the protein. In most cases having a stable linkage to the protein is beneficial because of the suitability for

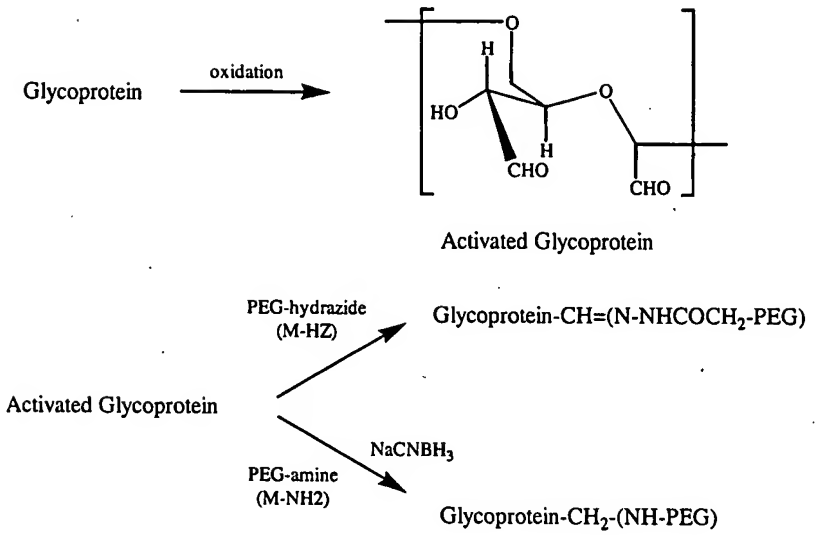

Fig. 7. Attachment of poly(ethylene glycol) to oxidized carbohydrates of glycoproteins.

long-term storage, easier purification and availability of prefilled syringes. It is also generally observed that stable linkages to a protein can reduce the activity, possibly due to the presence of the PEG chain at the active or binding site of the protein or steric crowding at the active or binding site. Also the PEG molecular weight has a direct impact on the activity; higher molecular weight PEG conjugates tend to have lower in vitro activity but have higher in vivo activity due to the improved pharmacokinetic profile [51]. The objective of most PEG conjugation techniques is to increase the circulation half-life without altering activity. In the development of PEG-Intron®, Enzon used a degradable linkage between the PEG and protein to improve the pharmacokinetic half-life but minimize loss of activity by releasing native interferon alpha-2b [52]. PEG-Intron is formed by conjugation of PEG-SC to interferon alpha-2b at low pH (around pH 5). The conjugation leads to a population of PEG conjugates coupled to His^{34} (Fig. 8). In this case, the PEG is coupled to the $N^{\delta 1}$ position of the imidazole ring in histidine to form a carbamate linkage and the PEG was found to be released from the protein over time. Note should be taken when comparing PEG-Intron to the branched $PEG_{40\ kDa}$-interferon alpha-2a conjugate (Pegasys®) that the PEG-Intron product has a higher in vitro activity compared to Pegasys, but the in vivo

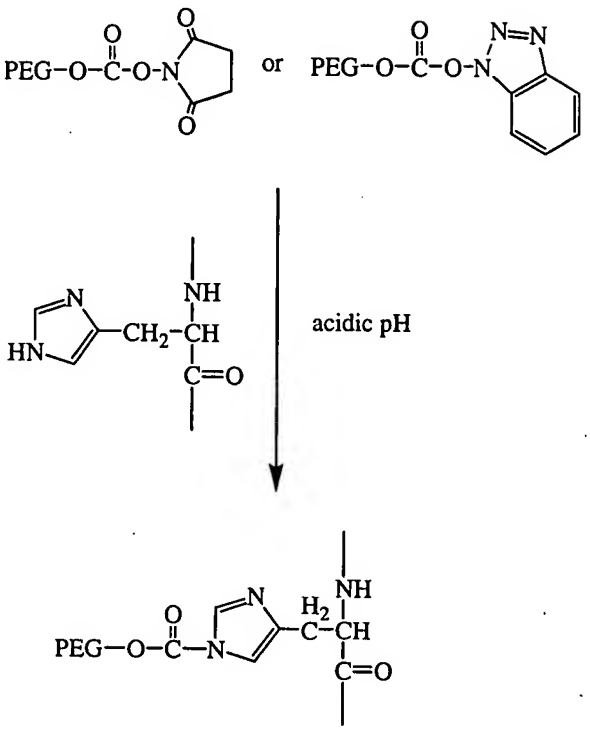

Fig. 8. Attachment of PEG-benzotriazole or PEG-succinimidyl carbonate to histidine residues on proteins.

activity of the Pegasys product is higher due to the superior pharmacokinetic profile [53,54].

An approach to regaining protein activity lost by PEGylation is the use of PEG chemistry that releases the native protein over time through enzymatic degradation, hydrolytic cleavage or reduction. The first such PEG reagent was PEG-succinimidyl succinate, described above. Other 'double ester' PEG reagents have been investigated by Roberts et al. to help control the release rates of the protein and regain activity over a period of time [55]. In this case, hydroxy acids are attached to carboxylic acids of PEG (carboxymethyl, propionic, or butanoic) to create a PEG acid that has an ester linkage between the hydroxy acid and PEG acid (Fig. 9). The terminal acid of the PEG derivative can then be activated and attached to α- and ε-amino groups of proteins. Regeneration of at least 60% of the native activity of lysozyme was recovered at physiological conditions of completely inactivated protein after release of the PEG from the protein. The problem with the double ester PEG reagents is that they release a protein that contains a 'tag' that could lead to immunogenicity of the protein.

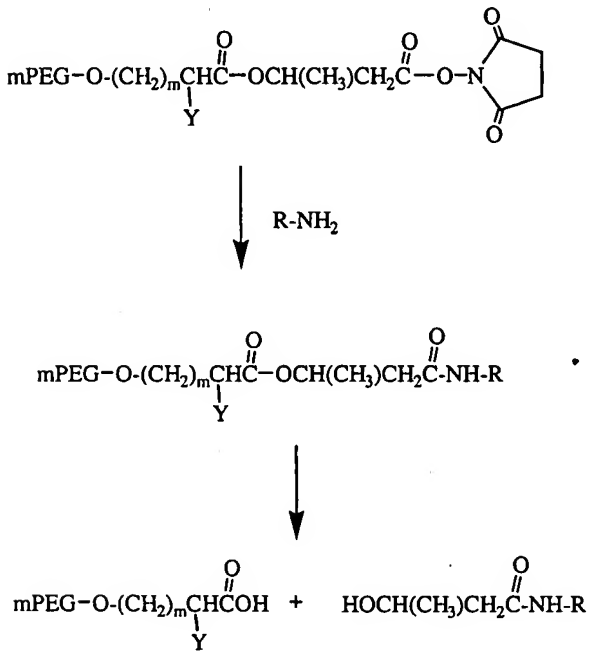

Fig. 9. Attachment and release of PEG-double esters from proteins (Y is an aliphatic or aromatic moiety).

To circumvent the loss of activity associated with some PEG modifications and the potential immunogenic nature of proteins released from PEG with a 'tag' as described above, reagents that release the native protein without 'tags' seem to be a better choice for protein modification. The first such reagent was PEG maleic anhydride used by Garman et al. to attach PEG to tissue plasminogen activator and urokinase (Fig. 10) [56]. Both of the conjugates regenerated the native protein under physiological conditions and had a 5–10× slower clearance rate than the native protein in the guinea pig.

Another example of a releasable PEG reagent was prepared by Bentley et al. [57]. In this work, mPEG phenyl ether succinimidyl carbonates and mPEG benzamide succinimidyl carbonates (Fig. 11) are used to conjugate to amino groups on lysozyme. Both conjugates regenerated the native protein under physiological conditions and the rate of release was controlled by the substitution position on the phenyl.

Greenwald et al. also synthesized a releasable PEG reagent that released native protein by a 1,6 elimination mechanism (Fig. 12) [58]. Again, the native protein was regenerated with nearly 100% of its bioactivity.

A further example of a releasable PEG was proposed by Zalipsky et al., which released the native protein by a mechanism other than hydrolysis [59]. The linkage as shown in Fig. 13 employs a *p*- or *o*-disulfide of a benzyl urethane. When subjected to mild reducing environments, such as that present in endosomal compartments of a cell, the original amine component is regenerated.

3.2.5. Heterobifunctional PEG chemistry

As applications of PEG chemistry have become more sophisticated, there has been an increasing need for heterobifunctional PEGs, which are PEGs bearing dissimilar terminal groups. Such heterobifunctional PEGs bearing appropriate functional groups may be used to link two entities where a hydrophilic, flexible, and biocompatible spacer is needed. Heterobifunctional PEG can be used in a variety of ways that includes linking macromolecules to surfaces (for immunoassays, biosensors or various probe applications), targeting of drugs, liposomes and viruses to specific tissues, liquid phase peptide synthesis and many others.

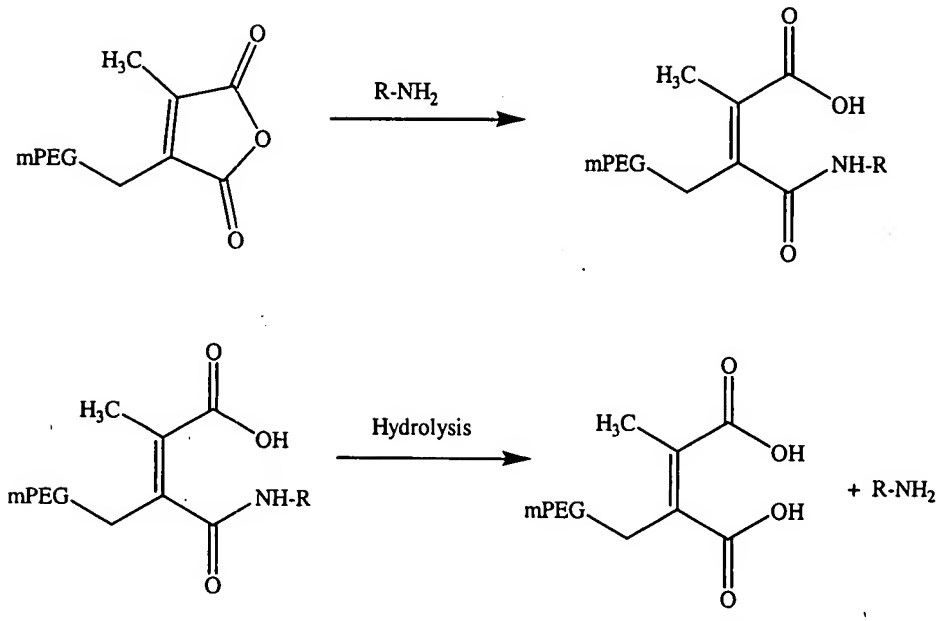

Fig. 10. Attachment and release of PEG-methylmaleic anhydride from amine containing drugs.

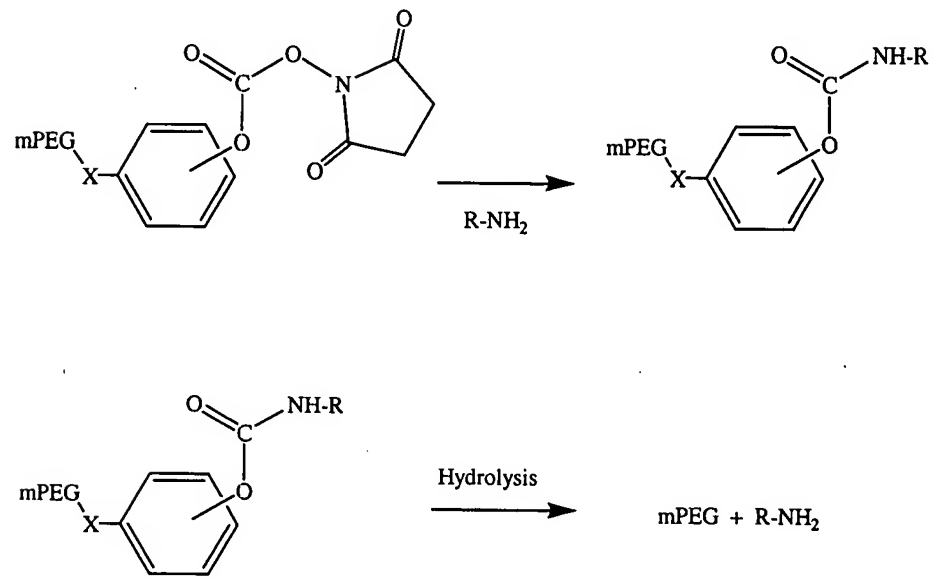

Fig. 11. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (hydrolysis mechanism) [X is O or –CONH–].

Several methods have been developed to synthesize heterobifunctional PEGs, each having its own advantages and disadvantages. Preferred end groups for heterobifunctional PEGs are NHS esters, maleimide, vinyl sulfone, pyridyl disulfide, amine, and carboxylic acids. The first such synthetic method

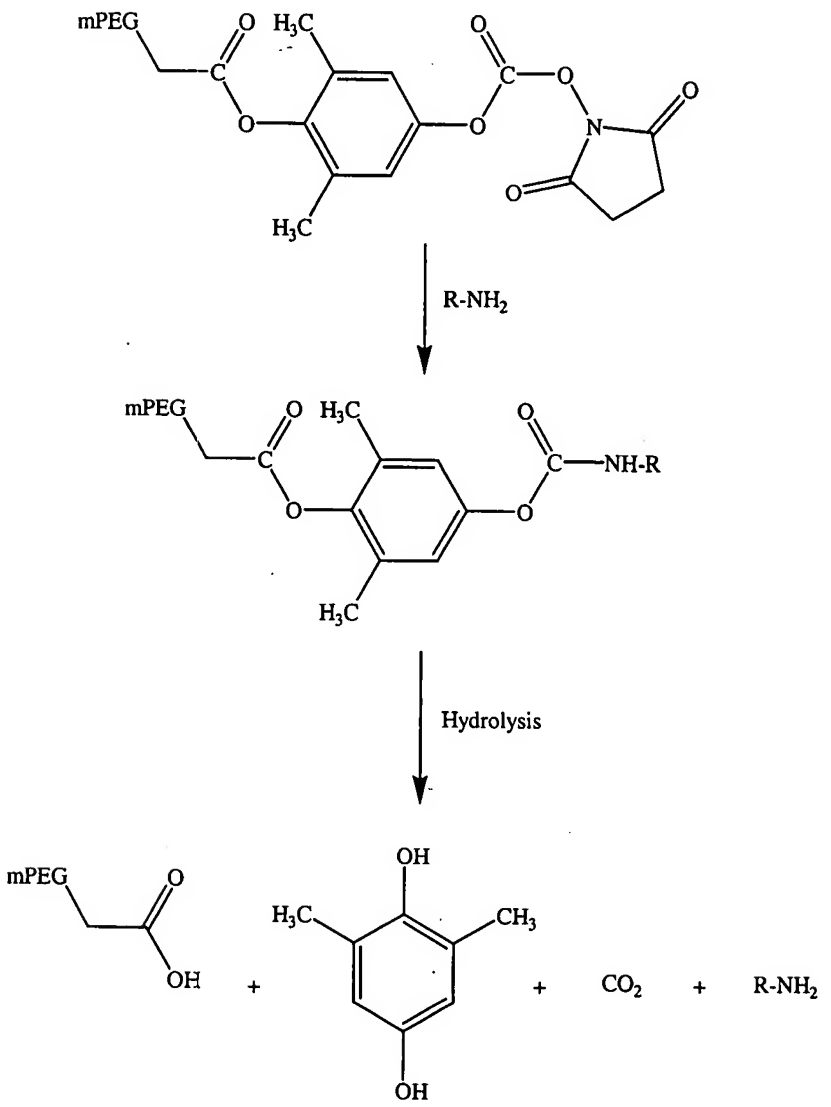

Fig. 12. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (1,6-elimination mechanism).

is to simply limit molar equivalents of each reagent. However, a distribution of substitution will result as shown below.

HO–PEG–OH

HO–PEG–COOH

HOOC–PEG–COOH.

A chromatographic approach was used by Zalipsky et al. to purify a PEG acid that was synthesized by reacting glycine with a PEG succinimidyl carbonate derivative [40]. Ion exchange chromatography was shown to be a very effective purification method to separate monoacid from diacid and unsubstituted PEG.

Bentley et al. prepared heterobifunctional PEGs by first initiating PEG polymerization with benzyl alcohol to produce benzyl PEG (Bz–PEG–OH) [60]. The hydroxyl group of the intermediate polymer is

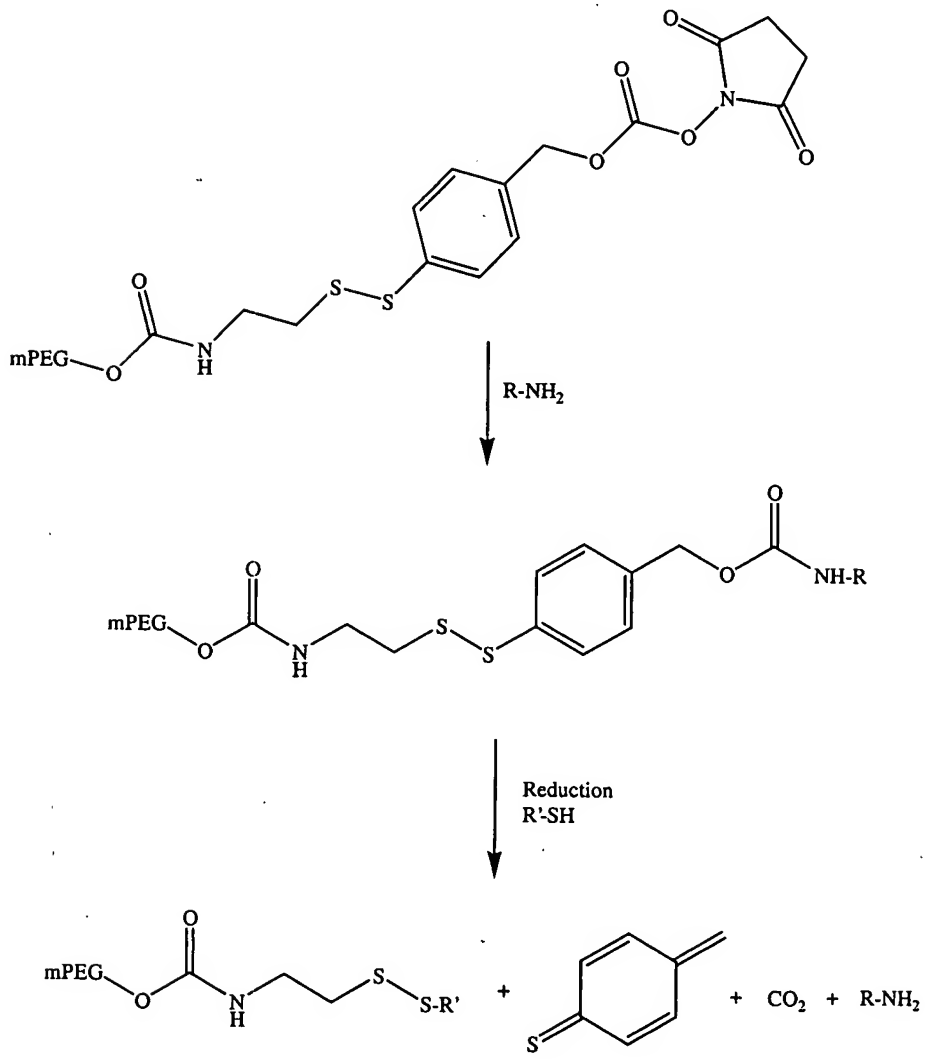

Fig. 13. Attachment and release of PEG phenyl NHS carbonates from amine containing drugs (reduction mechanism).

converted to a first reactive functional group. The benzyl group can then be removed by hydrogenolysis or hydrolysis, without chemically affecting the first functional group, thus making available a new terminal hydroxyl group for the addition of a second functional group.

A promising strategy for generating heterobifunctional PEGs is the polymerization approach. Initiating polymerization of ethylene oxide with an anion that ultimately becomes the end-group of PEG is the most direct route for heterobifunctional synthesis. Terminating the reaction with water generates a hydroxyl group at the second terminal end that can undergo activation by chemical methods. For example, this method has been used by Yokoyama et al. to prepare a PEG with a hydroxyl group at one terminus and an amino group at the other [61]. Cammas et al. have also used this method to prepare PEGs with an amino group on one terminus and a hydroxyl or methoxy group on the other [62]. It has also been used by Nagasaki et al. to prepare a PEG having a formyl group at one terminus and a hydroxyl group at the other [63]. This strategy also has its limits. Only those anions that are desirable as

end groups and suitable for initiating polymerization are useful for synthesis of heterobifunctional PEG by this route. This method is also limited by the fact that rigorous exclusion of water is necessary to prevent the formation of diol. This problem becomes more severe as PEG molecular weight increases. If these limits are resolved, generating heterobifunctional PEG reagents by anionic polymerization may be the most efficient and cost effective method.

3.3. PEG structures

In addition to the linear structure of the PEG molecule shown above, branched structures have proven useful for protein and peptide modification. The first branched PEG structure, 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine ($mPEG_2$-chlorotriazine), was based on a triazine core and synthesized by Inada et al. as shown in Fig. 2 [24].

Yamasaki et al. first synthesized a more useful branched PEG structure, based on a lysine core [64]. A highly purified branched PEG or PEG2 (Fig. 14A) was constructed by Veronese et al. using two linear PEG-BTC (or the related PEG-SC) chains linked to the α- and ε-amino groups of lysine [65]. This construct allows for a large molecular weight (upward of 60 kDa) and highly pure PEG to be synthesized with a single reactive end group. The standard preparation of PEG2 intermediate acid contains impurities. These impurities consist of unreacted PEG-BTC, a lysine residue that has only one PEG chain attached to one of the amino groups ('PEG1'), and 'PEG3', which is a linear PEG having a lysine at each end of a diactivated PEG impurity and two mPEG-BTC molecules coupled to the remaining two amino groups (thus PEG3 is a diacid). During aqueous work-up, the unreacted mPEG-BTC is converted back to mPEG-OH. Thus the reaction mixture contains a diacid, a zwitterion, a neutral mPEG, and the desired monoacid, which can be purified by careful ion-exchange chromatography. The acid of the lysine linker can be converted into a range of other derivatives including NHS esters, aldehydes, thiols and maleimide.

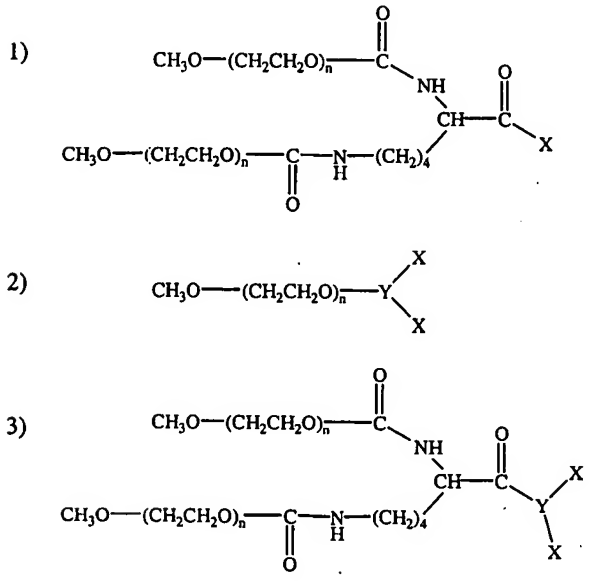

Fig. 14. Various PEG structures. (1) Branched PEG (PEG2), (2) linear forked PEG, and (3) branched forked PEG [Y is a group having a carbon branching moiety and X is a molecule, linker and/or a functional group].

PEG2 turns out to be a very exciting protein PEGylation reagent because of its unique characteristics when compared to linear PEGs. For example, PEG2 attached to proteins 'acts' much larger than a corresponding linear mPEG of the same M_w [64]. This structure of PEG also has the advantage of adding two PEG chains per attachment site on the protein, therefore reducing the chance of protein inactivation. Furthermore, the PEG2 structure is more effective in protecting proteins from proteolysis, reducing antigenicity and reducing immunogenicity [66].

Another branched PEG is the forked PEG. Instead of having a single functional group at the end of two PEG chains, as with PEG2, forked PEG has two reactive groups at one end of a single PEG chain or branched PEG (Fig. 14B,C). Harris et al. first synthesized a forked PEG by attaching to the terminus of a polymer backbone a single functional group of a trifunctional linker, such as serinol or β-glutamic acid [67]. The remaining proximal functional groups, which are linked to a central carbon atom, are able to react with two other molecules, which can be the same or different, to produce a PEG molecule that contains two molecules at a single terminus of the PEG chain.

Arnold et al. synthesized PEG compounds having terminal metal chelating groups, which consisted of

two free carboxylic acid or amino groups linked to a central nitrogen atom [68]. The PEG compounds were used to extract and precipitate proteins from solutions with the carboxylic acid or amino groups together with the nitrogen atom capable of forming ionic complexes with metal ions. Similar PEG compounds were synthesized by Martinez et al. to link hydroxyl containing moieties to the two terminal carboxylic acid groups to create a PEG-linked prodrug [69].

Forked PEG is useful for conjugating molecules where the benefit would be to bring two moieties in close proximity to one another, for example, dimerization of cell surface receptors to activate cellular mechanisms. Also, of interest is conjugating a F(ab)′ fragment of an antibody to the proximal reactive groups to produce a conjugate that closely resembles the structure of the full-length antibody. Another useful attribute of forked PEG is the increased loading capacity of small molecule pharmaceuticals.

4. Conclusions

The array of PEG chemistries reviewed here are among dozens being used for clinical development of PEGylated peptides and proteins. The transition from first-generation chemistries to second-generation chemistries is taking place at a rapid pace and future demands for PEG reagents will lead to new reagents for novel applications in the biopharmaceutical industry. Novel PEG chemistry for site-specific modification, as well as control of PK/PD parameters, will be synthesized when the needs arise. The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates make it to late phase clinical trials. Clearly, the scientific community is eagerly awaiting the results of the ongoing clinical trials to determine future product candidates. We expect that the approval of PEG-Intron for Hepatitis C and the FDA filing of Pegasys (PEG-IFN-alpha 2a), PEG-Neupogen® (PEG-G-CSF) and PEGVisomant (PEG-hGHra) will bring new life to a seasoned technology. What was thought to be a failing technology is now fulfilling its long recognized potential.

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